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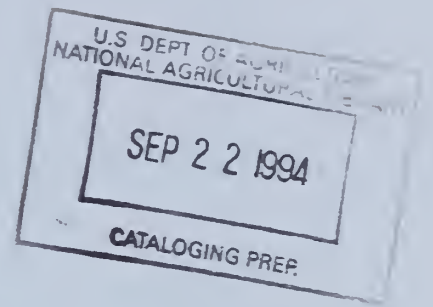
Food Safety
and Inspection
Service

Science and
Technology

Spring 1993

Analytical Chemistry Laboratory Guidebook

Food Chemistry



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FOREWORD

The new **Analytical Chemistry Laboratory Guidebook—Food Chemistry** is a reference book of regulatory methods for the analysis of meat and poultry products. Similar to its companion publication, the **Analytical Chemistry Laboratory Guidebook—Residue Chemistry**, which was published in 1991, this Guidebook has an enhanced, easy-to-read format. Wherever possible, quality assurance plans have been incorporated into each method.

We intend to revise and update the FSIS Analytical Chemistry Laboratory Guidebooks periodically. It is very important to us that our publications meet the needs of those who use them. Therefore we would welcome your comments and suggestions for improving the usefulness of the FSIS Analytical Chemistry Laboratory Guidebooks.

This publication is the result of joint efforts from the Food Safety and Inspection Service (FSIS) scientists in the Chemistry Division and the Technical Support Laboratories. We appreciate and acknowledge their contributions. We also gratefully acknowledge the commitment and strong support from the Training and Development Division, FSIS.

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CROSS-REFERENCE to 1987 Revision

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I. DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory

A fat or oil is saponified for thirty minutes, converting all of the fatty acids to a soap. The aqueous phase is extracted four times with ether and the ether extracts are added to a second separatory funnel. The combined ether extract containing unsaponifiable material is washed with alternate portions of KOH solution and water three times and repeatedly washed with water until the washings are no longer alkaline to phenolphthalein.

The ether extract is evaporated and dried and 1 mL of internal standard solution is added to the unsaponifiable residue. The solution is injected into a gas-liquid chromatograph equipped with a flame ionization detector and the cholesterol is quantified using the internal standard procedure.

Controversial samples are given additional cleanup using thin-layer chromatography (TLC). The entire sample is spotted and the plate developed. The sterol band is removed and the sterols eluted from the absorbent. The eluant is evaporated, dried, and dissolved in 1 mL of internal standard solution. Gas-liquid chromatography is again used to quantify the amount of cholesterol present. Confirmation is achieved by preparing the acetate derivative of the sterol and using gas-liquid chromatography for a qualitative determination.

2. Applicability

This procedure is applicable to the determination of animal fat as cholesterol in vegetable fats and oils.

I. DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

-
- a. TLC plates: Prepared from silica gel PF 254 + 366 or HF 254 + 366 (Brinkmann Instruments, Inc., or equivalent) or use precoated plates available as "Uniplat," precoated with silica gel HF 254 + 366, 500 μ m thick, No. 2112 (Analtech, Inc., 75 Blue Hen Dr., Newark, DE 19711) or "Quanta Gram," precoated with silica gel PQI-F with 366 nm phosphor, 500 μ m thick, No. 2026 (Quantum Industries, 341 Kaplan Dr., Fairfield, NJ 07006).
 - b. Thin layer plate scraper: Optional—adapt from sealing tube with fritted disk (Corning Glassworks, #39580, or equivalent).
 - c. Separatory funnel: 125 mL.
 - d. 4-dram vials with aluminum-lined caps.
 - e. Saponification flask (200 mL Erlenmeyer flask with standard taper 24/40 outer joint).
 - f. 250 mL separators.
 - g. 250 mL beaker.
 - h. 25 mL volumetric flask.
-

2. Instrumentation

Gas chromatograph: Equipped with flame ionization detector and 1 mv strip-chart recorder. Temperatures 220-250° C (column), 240-270° C (detector and flash heater); flow rates N₂ (ultra high purity grade) 20-25 psi to elute cholesterol in 8-12 min; H₂ ca. 40-45 mL/min; air 300-340 mL/min. Electrometer sensitivity 1×10^{-9} amp full-scale deflection with 1 mv recorder. Adjust electrometer sensitivity so that 1.5 μ g cholesterol gives ca. 50% deflection. Repeat injections until constant peak heights are obtained on successive injections of identical volumes of standard mixture.

I. DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

**Reagent and
Solution List**

-
- a. Ethyl acetate (EtOAc): Distilled in glass or equivalent.
 - b. Chloroform: Distilled in glass or equivalent.
 - c. Ethyl ether: Anhydrous 0.01% alcohol.
 - d. Petroleum ether: Distilled in glass, bp 30-60° C or equivalent.
 - e. KOH solution (3 + 2).
 - f. Ethyl alcohol: Reagent grade.
 - g. Pyridine: Reagent grade.
 - h. Acetic anhydride: Reagent grade.
 - i. GLC column packing.
 - i. Stationary phase. JXR, or OV-1 or OV-101 dimethyopolysiloxane, or OV-17 or OV-22 methylphenylpolysiloxane.
 - ii. Support. 100-120 mesh Gas-Chrom Q. Commercially prepared packing of 1 or 3% stationary phase available from Applied Science Laboratories, Inc., or Supelco, Inc.
 - j. Phenolphthalein indicator.
-

I. DETERMINATIVE METHOD

D. STANDARDS

1. Sources

-
- a. Cholesterol, cholestane—Applied Science Laboratories, Inc.
 - b. β -Sitosterol—Aldrich Chemical Co., Inc.
 - c. Cholesteryl acetate—ICN Pharmaceuticals, Inc., Life Sciences Group.
-

2. Preparation of Standards

- a. Cholesterol standard solution: 1.2 $\mu\text{g}/\mu\text{L}$. Weigh 60.0 mg cholesterol standard into 50 mL volumetric flask and dilute to volume with EtOAc.
 - b. Cholestane standard solution: 0.4 $\mu\text{g}/\mu\text{L}$. Weigh 40.0 mg cholestane standard into 100 mL volumetric flask and dilute to volume with EtOAc.
 - c. Cholestane internal standard solution: 0.2 $\mu\text{g}/\mu\text{L}$. Dilute 10.0 mL standard solution b to 20.0 mL with EtOAc.
 - d. Cholestane-cholesterol standard mixture: 0.2 μg cholestane and 0.6 μg cholesterol/mL. Mix equal volumes of solutions a and b.
 - e. β -Sitosterol standard solution: 3 $\mu\text{g}/\mu\text{L}$. Weigh 30.0 mg β -sitosterol standard into 10 mL volumetric flask and dilute to volume with EtOAc. Commercial material is mixture of campesterol (earlier eluting component) and β -sitosterol.
 - f. Cholesterol- β -sitosterol standard mixture: 0.6 μg cholesterol and 1.5 μg β -sitosterol/mL. Mix equal volumes of solutions a and e.
 - g. Cholesteryl acetate standard solution: 0.5 $\mu\text{g}/\mu\text{L}$. Weigh 30.0 mg cholesteryl acetate standard into 50 mL volumetric flask and dilute to volume with EtOAc.
-

I. DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE

1. Column Preparation

- a. Pack glass column, 6' (1.8 m) \times 4 mm id, with commercially available 1-3% stationary phase on 100-120 mesh Gas-Chrom Q or dissolve 0.4-1.2 g polysiloxane in 200 mL toluene or CH_2Cl_2 -toluene (1 + 1). Heat to dissolve; polysiloxane dissolves slowly in solution mixture. Add solution to 40 g Gas-Chrom Q and let stand 10 min with occasional gentle stirring. Dry in rotary evaporator held in 50° C bath or heat on steam bath with occasional gentle stirring and remove residue solution in vacuum oven at 50° C.

CAUTION: Siloxanes are toxic. Wear disposable gloves and use effective fume removal device when handling.

- b. Conditioning of column. Heat 8 hr at 260° C with ca. 5-10 psi N_2 flowing through column. Shut off pressure, raise temperature to 290° C, and continue heating \geq 8 hr. Reduce temperature to 260° C, adjust N_2 to 5-10 psi, and heat additional 12-24 hr.
- c. Performance. Chromatograph ca. 2 μL cholesterol- β -sitosterol standard mixture to determine retention times and resolution of column. Minimum of 1600 theoretical plates is required for cholesterol peak; theoretical plates = $(L/B)^2 \times 16$, where L = cm cholesterol peak from injection point, and B = cm triangulated base width of cholesterol peak. In addition, separation of cholesterol and campesterol peaks, expressed as peak resolution, should be 2.2. Peak resolution = $2D/(B + P)$, where D = distance in cm between cholesterol and campesterol peak maximum, B = triangulated base width of cholesterol peak, and P = triangulated base width of campesterol peak. Determine peak resolution on sample having ca. equal amounts cholesterol and campesterol (ca. equal peak areas); sample injected should give peak heights 25-30% of Full Scale Deflection (FSD).

2. Sample Extraction

- a. Accurately weigh 2-2.5 g vegetable oil into saponification flask (200 mL Erlenmeyer flask with standard taper 24/40 outer joint is recommended).
- b. Add 25 mL ethyl alcohol and 1.5 mL (3 + 2) KOH solution. Saponify by boiling 30 min, with occasional swirling, on steam bath under reflux air condenser. (No loss of alcohol should occur during saponification.)
- c. Transfer alcohol-soap solution while still warm to 250 mL separator, using total of 50 mL H_2O . Rinse saponification flasks with 50 mL ether and add ether to separator.
- d. Shake vigorously and let layers separate and clarify.
- e. Drain lower layer back into saponification flask and pour ether layer through top into second separator containing 20 mL H_2O . Rinse pouring edge with ether, adding rinsings to second separator.
- f. Extract soap solution in the saponification flask with two 50 mL portions of ether in the same manner.
- g. Make a total of 4 extractions for marine oils or other oils of high unsaponifiable content.

I. DETERMINATIVE METHOD

F. ANALYTIC PROCEDURE (Continued)

-
- h. Rotate combined ether extracts gently with the 20 mL H₂O. (Violent shaking at this stage may cause troublesome emulsions.)
 - i. Let layers separate and drain aqueous layer.
 - j. Wash with two additional 20 mL portions H₂O, shaking vigorously.
 - k. Wash ether solution three times with alternate 20 mL portions ca. 0.5N aqueous KOH and H₂O, shaking vigorously each time.
 - l. If emulsion forms during washing, drain as much aqueous layer as possible, leaving emulsion in separator with ether layer, and proceed with next washing.
 - m. After third KOH treatment, wash ether solution successively with 20 mL portions H₂O until washings are no longer alkaline to phenolphthalein.
 - n. Quantitatively transfer the ether extract to a 250 mL beaker and evaporate to dryness under nitrogen.
 - o. Dissolve unsaponifiable in 4-5 mL CHCl₃ and mix on vortex mixer for about 30 sec.
 - p. Transfer to 25 mL volumetric flask, repeat with four more aliquots of CHCl₃, adjust to mark, and store in freezer.
-

3. Determination

For qualitative determination, inject 10 μ L of solution p and compare with 4 μ L injection of standard cholesterol (item D.2.a). If this qualitative test indicates the presence of animal fats in vegetable oil, evaporate the entire remaining solution to dryness under N₂ and store in freezer. Proceed to step F.4.

4. Isolation of Sterols by TLC

- a. Preparation of plates.
 - i. Align 5 matching 20 \times 20 cm glass plates on mounting board; just before coating, wipe plates with tissue dampened with alcohol to remove any dust or fingerprints.
 - ii. Adjust applicator to deliver 0.5 mm-thick layer.
 - iii. Weigh 45 g silica gel into 500 mL Erlenmeyer flask, add 130 mL H₂O, shake vigorously 25-30 sec, and pour into applicator.
 - iv. Immediately coat plates with silica gel suspension and let plates rest undisturbed until jelled (0.5-1 hr).
 - v. Dry coated plates 2 hr at 110° C and store in desiccating cabinet until just before use.
- b. Thin Layer Chromatography
 - i. Line developing chamber with blotting paper and add 100 mL ether-pet ether (1 + 1) to chamber. Cover chamber and equilibrate 2 hr.

I. DETERMINATIVE METHOD

F. ANALYTIC PROCEDURE (Continued)

-
- ii. Draw line across plate 17 cm from bottom and ca. 1 cm from each side. Spot 10 μ L β -sitosterol standard solution (item D.2.e) at a point 2 cm from the bottom edge and 3 cm from one side of the plate.
 - iii. Dissolve unsaponifiable matter in 200 μ L CHCl_3 and spot entire sample in 10 μ L portions on imaginary line 2 cm from bottom edge of plate so that spot centers are 0.75 cm apart.
 - iv. Rinse vial with ca. 100 μ L CHCl_3 and spot rinse solution in equal portions on top of sample spots.
 - v. Immediately insert plate into equilibrated chamber (position plate to expose coated surface to maximum chamber volume). Cover chamber and seal with tape.
 - vi. Withdraw plate from chamber when solvent front reaches 17 cm stop line.
 - vii. Evaporate solvent and view plate under long-wave UV light in darkened room.
 - viii. Mark off sterol band (same R_f , 0.2-0.3, as β -sitosterol standard) with needle and remove sterol band as follows (do not remove β -sitosterol standard): Scrape off sterol band with square end of stainless steel spatula into 100 mL beaker and transfer with 20 mL CHCl_3 to 70 mm top diameter funnel containing folded 12.5 cm diameter filter paper.

Extract sterols with five 10 mL portions CHCl_3 and evaporate combined filtrate to near dryness on steam bath under nitrogen.

NOTE: Alternatively, remove sterol band with TLC plate scraper, elute sterols from silica gel with 70 mL CHCl_3 (fourteen 5 mL portions), and evaporate solvent to near dryness on steam bath under nitrogen.

5. Gas Chromatography of Sterols

-
- a. Pipet 1.0 mL cholestane internal standard solution (item D.2.c) into 4-dram vial containing extracted sterols, rotate vial to wash down sides with internal standard solution, and swirl to dissolve sterols.
 - b. Inject 2 μ L sample at least in duplicate. Repeat with 2 μ L cholestane-cholesterol if peak height of sample is 60% full-scale deflection, add additional 1.0 mL cholestane internal standard solution to sample, and chromatograph sample and standard mixture as above.
-

I. DETERMINATIVE METHOD

G. CALCULATIONS

1. Procedure

Measure cholestane and cholesterol peak heights in nm. Calculate mg cholesterol/100 g sample, correcting for internal standard as follows:

$$\text{i. mg cholesterol/100 g} = (H_i/H_s) \times (C_s/C_i) \times (S_s/S_i) \times (Q_i/Q) \times 100$$

H_i and H_s = ht (mm) cholestane and cholesterol peaks respectively in standard mixture.

S_s and S_i = ht (mm) cholesterol and cholestane peaks respectively in sample.

C_s and C_i = μg cholesterol and cholestane/ μL respectively in the standard mixture.

Q_i = μg cholestane/ μL in sample.

Q = mg sample/ μL .

$$\text{ii. Percent animal fat} = \frac{(\text{mg cholesterol/100 g} - 1.5 \text{ mg/100 g})}{82.0 \text{ mg/100 g}} \times 100$$

1.5 mg/100 g = cholesterol-like component due to edible vegetable oils

NOTE: Some vegetable oils, notably palm oil, or those containing mono and diglycerides derived from animal fat, may have higher levels of this component present. In that case, a sample of the oil will be required prior to processing to determine the amount of the component (mg/100 g).

82.0 mg/100 g = the average cholesterol content of beef and pork fat

2. Reference

Official Methods of Analysis of the Association of Official Analytical Chemists, 15th Edition.

I. DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Animal Fat in Vegetable Fats and Oils		
2. Protective Equipment	Safety glasses, plastic gloves, heat resistant gloves, and lab coat.		
3. Procedure Steps		<u>Hazards</u>	<u>Recommended Safe Procedures</u>
	C. Reagents		
	Ethyl acetate	Skin, eye, and respiratory irritant.	This entire procedure should be performed in an efficient fume hood. The surrounding area should be well-ventilated. Never use hot plates for evaporation and concentration steps. Ethyl ether lots should also be checked for peroxides.
	Chloroform	Volatile and may induce nausea. Carcinogenic.	
	Ethyl ether Petroleum ether	Extremely flammable. May induce nausea or unconsciousness.	
	KOH solution (3 + 1)	Strong corrosive. May cause irreversible brain damage.	
4. Disposal Procedures	Ethyl and petroleum ethers and ethyl acetate.	See above.	Evaporate in a fume hood.
	Chloroform.	See above.	Store in waste chlorinated solvent container for disposal by contractor or in-house specialist.
	KOH solution.	See above.	May be partially neutralized before flushing down disposal sink with large amounts of water.

II. CONFIRMATORY METHOD

Confirmatory Test

The presence of cholesterol may be confirmed by GLC of sterol acetates. After determining cholesterol by GLC, evaporate sample to dryness on steam bath under nitrogen. Cool and add 3 mL pyridine and 1 mL acetic anhydride. Cap vial, swirl on steam bath until sterols dissolve, and continue heating on steam bath 1 hr. Evaporate using nitrogen stream until no odor of pyridine is detected. Chromatograph sterol acetates and cholesteryl acetate standard solutions and compare retention times of sample and cholesteryl acetate peaks.

ANTIOXIDANTS

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DETERMINATIVE METHOD

A. INTRODUCTION

Theory

The prevention of oxidative degradation in fats may be controlled by the use of synthetic phenolic antioxidants. Specific acceptable antioxidants in the U.S., and their respective limits in meat, are found in the Regulations. This test detects the presence in fats, oils, and shortenings, and quantitates the amount of, 2- and 3-tert-butyl-4-hydroxyanisole (BHA); tert-butyl-hydroquinone (TBHQ); 3,5-di-tert-butyl-4-hydroxytoluene (BHT); 2,6-di-tert-butyl-4-hydroxymethylphenol (IonoX-100); 2,4,5-trihydroxybutyrophenone (THBP); propyl gallate (PG); octyl gallate (OG); dodecyl gallate (DG); and nordihydroguaiaretic acid (NDGA). The antioxidants are extracted from fat with hexane saturated with acetonitrile and partitioned into acetonitrile saturated with hexane, concentrated under vacuum, and determined by reverse phase gradient elution high performance liquid chromatography (HPLC) with detection at 280 nm. The mobile phase is water-acetonitrile with 5% acetic acid. With this system, only IonoX 100 and OG are not resolved. Another mobile phase is used to resolve these two antioxidants.

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

-
- a. Food chopper, suitable for grinding processed products.
 - b. Steam bath.
 - c. 125 mL and 250 mL separatory funnels.
 - d. 250 mL round-bottom flasks.
 - e. Rotary flask evaporator—Rinco, or equivalent, with $\leq 40^{\circ}$ C water bath.
 - f. Disposable pipets.
 - g. 10 mL stoppered graduated cylinders.
 - h. Filter paper—coarse to fit into apparatus i.
 - i. Powder funnels—to fit into 250 mL beakers.
 - j. 50 mL, 150 mL, and 250 mL beakers.
 - k. Balance top loader—Mettler P1200 or equivalent.
 - l. 50 mL and 100 mL volumetric flasks.
 - m. 25 mL pipets.
 - n. 1 mL pipets.
-

2. Instrumentation

- a. Liquid chromatograph—Waters Model No. 244, equipped with Model No. 660 solvent programmer operated at ambient conditions at 2 mL/min with Model No. U6K sample injector valve, or equivalent. A suitable uv detector with sensitivity range 0.01-2 absorbance units full scale (AUFS), measuring absorbancies at 280 nm.
 - b. Chromatographic columns:
 - i. Analytical column— $4.6 \times 250 \mu\text{m}$ id stainless steel column packed with $10 \mu\text{m}$ Li Chrosorb RP-18 (Altex Scientific Inc.), or equivalent.
 - ii. Stainless steel guard column— $42 \times 2.6 \text{ mm}$ id, packed with 38 mm mean diameter Li Chrosorb RP-2 (E Merck, Darmstadt (GFR), or equivalent, placed in line just before the analytical column, using zero dead volume fittings.
-

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

**Reagent and
Solution List**

-
- a. Acetonitrile—Distilled-in-glass grade, or equivalent.
 - b. 2-propanol—Distilled-in-glass grade, or equivalent.
 - c. Hexane—Distilled-in-glass grade, or equivalent.
 - d. Methanol—Distilled-in-glass grade, or equivalent.
 - e. Acetic acid—A.R. grade.
 - f. Mobile Phases
 - i. Distilled water—Millipore HPLC-grade, bottled. Add 5% v/v, A.R. grade acetic acid.
 - ii. Acetonitrile—Use spectrograde or quality suitable for HPLC. Add 5% v/v A.R. grade acetic.
 - iii. Methanol—Use spectrograde or quality suitable for HPLC. Add 5% v/v A.R. acetic acid.
 - g. Extracting solvent—Saturate hexane with acetonitrile by shaking together for 2 min in a separatory funnel.
 - h. Partitioning solvent—Use acetonitrile layer from g.
 - i. Anhydrous sodium sulfate, reagent grade.
-

DETERMINATIVE METHOD

D. STANDARDS

1. Source

-
- a. BHA, BHT, TBHQ, Ionox 100, THBP, and PG (Available from Polyscience Corporation, Niles, IL 60648).
 - b. OG and DG (Available from Naarden, The Netherlands).
 - c. NDGA (Food Chemical Codex Reference Standard).

Equivalent sources may be used for any of the above.

2. Preparation of Standards

Standard Solution—Prepare all solutions with 2-propanol-acetonitrile (1 + 1).

- a. Stock Solution I—1 mg/mL. Accurately weigh and transfer 50 mg each of PG, TBHQ, NDGA, OG, and BHT into one 50 mL volumetric flask; dissolve, dilute to volume, and mix.
 - b. Stock Solution II—1 mg/mL. Accurately weigh and transfer 50 mg each of THBP, BHA, Ionox 100, and DG into one 50 mL volumetric flask; dissolve, dilute to volume, and mix.
 - c. Standard Solution I—0.01 mg/mL (10 μ g/mL). Pipet 1 mL stock solution I into 100 mL volumetric flask; dilute to volume and mix.
 - d. Standard Solution II—0.01 mg/mL (10 μ g/mL). Pipet 1 mL stock solution II into 100 mL volumetric flask; dilute to volume and mix.
 - e. Standard Solution I & II—0.01 mg/mL (10 μ g/mL). Pipet one mL each of stock solutions I and II into 100 mL volumetric flask; dilute to volume and mix.
-

3. Storage Conditions

Refrigerate all antioxidant solutions out of direct light.

DETERMINATIVE METHOD

E. SAMPLE HANDLING

Sample Preparation

-
- a. *Processed Products and Fats*—Pass sample through food chopper with suitable quantity of anhydrous sodium sulfate to combine with moisture in sample. Do not use sodium sulfate if sample is fat only. Place chopped sample (sufficient to yield 10-25 g fat) in a beaker. Slurry with hexane saturated with acetonitrile and filter through coarse filter paper into 250 mL beaker. Evaporate solvent on steam bath. Go to step F.1.a.
 - b. *Liquid Oils*—Accurately weigh 20.0 g oil into 50 mL beaker and quantitatively transfer to 100 mL volumetric flask, rinsing beaker with hexane saturated with acetonitrile. Dilute to volume with hexane saturated with acetonitrile and mix. Go to step F.1.c.
-

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE

1. Determination

- a. Accurately weigh 10 g lard, shortening, or extracted fat into 150 mL beaker. Add ca. 30 mL hexane saturated with acetonitrile and dissolve sample, heating gently if necessary.
- b. Quantitatively transfer dissolved fat to 100 mL volumetric flask, rinsing beaker with hexane saturated with acetonitrile; dilute to volume and mix.
- c. Pipet 25 mL aliquot into 125 mL separatory funnel and partition with three 50 mL portions of acetonitrile saturated with hexane. If emulsions form, break by holding separatory funnel under hot tap water for 5-10 sec.
- d. Collect partitions in 250 mL separatory funnel and let combined partitions slowly drain into 250 mL round-bottom flask to aid removal of hexane/fat droplets.
- e. Evaporate to 3-4 mL, using rotary evaporator with $\leq 40^{\circ}$ C water bath.

NOTE: Evaporation should be accomplished in < 10 min to avoid loss of TBHQ by oxidation. A good water aspirator is mandatory.

- f. Using disposable pipet, transfer acetonitrile/fat droplet mixture to 10 mL stoppered graduated cylinder. Rinse flask and disposable pipet with small portions of nonhexane-saturated acetonitrile, transferring rinsings to graduated cylinder with the pipet until 5 mL are collected. Rinse flask and disposable pipet with small portions of 2-propanol, transferring all rinsings to graduated cylinder until exactly 10 mL is collected. Mix contents of the cylinders.
- g. Using sample injector, inject in duplicate 20 μ l from graduated cylinder into instrument with column as described in apparatus B.1.a and b. Set range to 0.05 or 0.1 AUFS. For sample peaks off scale, accurately dilute sample solutions with 2-propanol-acetonitrile (1 + 1). Inject appropriate amount of standard solution D.2.c and/or d.
- h. Use linear gradient, programmed from 30% reagent C.f.ii in C.f.i to 100% C.f.ii over 10 min, with 4 min hold at 100% C.f.ii at 1 mL/min. Increase flow rate to 4 mL/min at 100% C.f.ii for 5 min to elute nonpolar lipids. Return to 30% C.f.ii over 1 min at 1 mL/min and let baseline, pressure, and solvent composition stabilize (ca. 10 min). Back pressure of ca. 1500 psi is attained at 2 mL/min at 30% acetonitrile in water and 2300 psi at 4 mL/min at 100% acetonitrile.
- i. If presence of Ionox-100 or OG is indicated, identify and quantitate using reagents C.f.i and C.f.iii to 100% C.f.iii over 10 min, with 4 min hold at 100% C.f.iii at 1 mL/min. Increase flow rate to 4 mL/min at 100% C.f.iii for 5 min. Return to 35% C.f.i in C.f.iii over 1 min at 2 mL/min and let baseline, pressure, and solvent composition stabilize (ca. 10 min). (NOTE: If both ionox and OG are present, accurate quantitation may not be possible.)

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE (Continued)

2. Identification and Quantification

Identify peaks as described in Figure 1 and Table 1. Each analytical system may vary slightly.

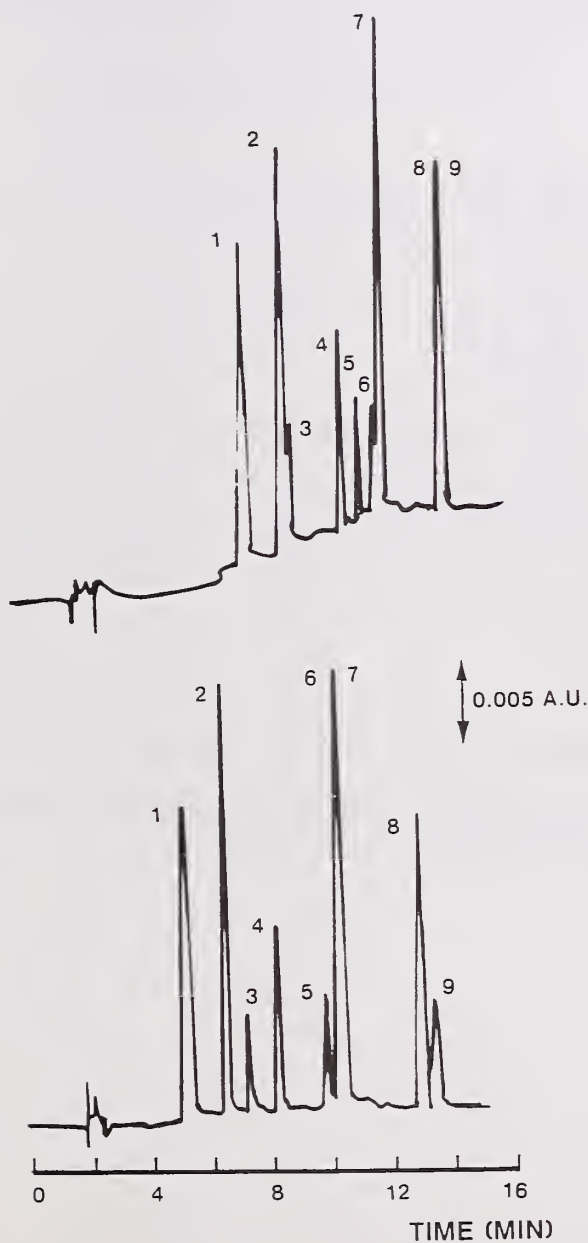


Figure 1—Chromatographic separation of antioxidant standards, ca. 80 ng each: 1, PG; 2, THBP, 3, TBHQ; 4, NDGA; 5, BHA; 6, Ionox-100; 7, OG; 8, DG; 9, BHT.

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE (Continued)

3. Table 1

Upper chromatogram, reagent C.f.i, 5% (v/v) acetic acid in water, reagent C.f.iii, 5% (v/v) acetic acid in methanol with linear gradient of 35% C.f.i in C.f.iii to 100% C.f.iii over 10 min; lower chromatogram, reagent C.f.i, 5% (v/v) acetic acid in water, reagent C.f.ii, 5% (v/v) acetic acid in acetonitrile with linear gradient of 30% C.f.ii in C.f.i to 100% C.f.ii over 10 min.

Retention times and Detector Response for Antioxidants

Antioxidant	Retention time, min ^a		
	Water-Acetonitrile	Water-Methanol	% FSD for 80 ng ^b
PG	5.1	7.4	41
THBP	6.3	8.6	56
TBHQ	7.1	8.9	13
NDGA	8.1	10.6	25
BHA	9.6	11.2	15
Ionox-100	10.0	11.7	9
OG	10.0	11.9	49
DG	12.7	13.9	39
BHT	13.1	13.9	11

^a Retention time of unretained compound (NaNO₃) is 1.4 min.

^b Percent full scale deflection (FSD) at 0.05 absorbance unit full scale with 5% acetic acid acetonitrile-water gradient.

I. DETERMINATIVE METHOD

G. CALCULATIONS

1. Procedure

Determine average peak height or average peak area of antioxidant containing sample from duplicate injections, average peak height or average peak area of antioxidant standard from duplicate injections, and calculate concentration of antioxidant as follows:

$$\text{Antioxidant, ppm} = (C_s/R_s) \times (R_x/W_x) \times D$$

$$\% \text{ Antioxidant in fat} = (\text{ppm}) 10^{-4}$$

where:

R_x = average sample peak height or average sample peak area

R_s = average standard peak height or average standard peak area

C_s = concentration of standard in $\mu\text{g/mL}$

W_x = weight of sample in $\mu\text{g/mL}$ in 10 mL final extract

D = dilution factor if solution injected is diluted.

R_x and R_s must be in the same units, i.e., height or area.

2. Reference

Page, B. Denis, *J. Assoc. Off. Anal. Chem.* (Vol. 62, No. 6. 1979), (Vol. 66, No. 3, 1983).

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Determination of Antioxidants in Rendered Fat from Processed Products, Lards, Shortenings, and Oils.		
2. Required Protective Equipment	Safety glasses, plastic gloves, lab coat.		
3. Procedure Steps		<u>Hazards</u>	<u>Recommended Safe Procedures</u>
	a. Processed products and fats pass sample through food chopper—etc. Slurry with hexane saturated with acetonitrile and mix.	Hexane—skin irritant and flammable	Procedures should be performed in an efficient fume hood. Hot plates or other electrical heating devices should not be used in the proximate area.
	b. Evaporate to 3-4 mL using rotary evaporator with $\leq 40^{\circ}\text{C}$ water bath.	Implosion	Check flasks for defects and use protective shield. Insure that water aspirator or vacuum system is working properly.
4. Disposal Procedures	Amount of organic solvent in the injection container (10 mL) can be easily evaporated.	Organic vapors	Warm on steam bath fume hood. After vapors have been removed, pour fatty waste into a plastic bag, freeze, and discard with other solid waste.
	Extracted processed product and fat waste.		

**BENZOIC ACID, SORBIC ACID, AND FOUR PARABENS IN
MEAT AND NONMEAT PRODUCTS BY HPLC**

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I. DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory

The antimicrobial preservatives, benzoic acid, sorbic acid, and parabens, are not permitted in fresh meat products or in seasoning mixtures used in the formulation of these products.

Ten grams of thoroughly comminuted meat samples or 2.0 g seasoning mixture are extracted with 70% ethanol. After filtration, extracts are analyzed by reverse phase liquid chromatography, using a 254 or 280 nm UV detector.

I. DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

-
- a. Magnetic stirrer—Variable-speed, Nuova 7 Thermolyne (Sybron Corp., Dubuque, IA 52001), or equivalent.
 - b. Stirring bar—Plastic-coated, magnetic, 1" long.
 - c. Filter paper—Reeve Angel grade 802 (Sargent-Welch Scientific Co., Skokie, IL 60077), fast-filtering and medium porosity (18.5 cm), or equivalent.
 - d. Solvent clarification kit—With Durapore filters (0.4 μ m, 47 mm), but without pump and filtering flask (Waters Associates, Milford, MA 01757).
 - e. Evaporator—N-Evap Model III (Organomation Associates, Inc., South Berlin, MA 01549), or equivalent.
 - f. Sample filtration apparatus—Stainless steel Swinney filter holder, with 0.45 μ m, Fluoropore filters (Millipore Corp., Bedford, MA, 01730), or equivalent.
 - g. pH meter—Fisher Accumet, Model 210 (Fisher Scientific Co., Pittsburgh, PA 15219), or equivalent.
-

2. Instrumentation

-
- a. Liquid chromatograph—Waters Associates, Model 244 Liquid Chromatograph, equipped with 2 pumps, Model M-6000A (one designated as pump A and the other as pump B), Model 440 absorbance detector, Wisp 710B sample processor, and Model 730 data module, or equivalents. Strip chart recorder, OmniScribe Recorder Model 35217-1 (Houston Instrument, Austin, TX 77041), or equivalent.
 - b. Column—15 cm \times 3.9 mm id, Nova-pak C₁₈ reverse phase packing, 5 μ m, spherical silica (Waters Associates), or equivalent.
-

I. DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and
Solution List

- a. Absolute ethanol—200 proof (U.S. Industrial Chemicals, Inc., Division of National Distillers Products Corp., New Orleans, LA).
 - b. Methanol—Distilled in glass, UV grade (Burdick and Jackson Laboratories Inc., Muskegon, MI 49442), or equivalent.
 - c. Glacial acetic acid—ACS grade.
 - d. Water—Low organic content and specific resistance ≥ 10 megaohms/cm to avoid problems with extraneous interferences, Milli-Q Water Purification System (Millipore Corp.), or equivalent.
 - e. Ammonium acetate—LC grade (Fisher Scientific Co.).
 - f. Hydrochloric acid—ACS grade.
 - g. Benzene—Thiophene-free ACS grade (Mallinckrodt, Inc., St. Louis, MO 63134).
 - h. LC mobile phase.
 - i. Buffer solution—15% (w/v) aqueous ammonium acetate and 15% (v/v) aqueous acetic acid. Into 1 L graduated cylinder, add 15 g ammonium acetate and 15 mL glacial acetic acid. Dilute to volume and mix well to dissolve ammonium acetate. Filter resultant aqueous acidic solvent (pH 4.50) through Durapore filter into 2 L filtering flask. Degas solvent under vacuum. Use solvent for pump A of the liquid chromatograph.
 - ii. Methanol—LC grade, filter and degas. Use solvent for pump B of liquid chromatograph.
-

I. DETERMINATIVE METHOD

D. STANDARDS

1. Preparation of Standards

Mixed standard solution—2.0 mg/mL benzoic acid, 0.08 mg/mL sorbic acid, and 0.20 mg/mL each methyl, ethyl, propyl, and butyl parabens. Weigh exactly 400.0 mg benzoic acid, 16.0 mg sorbic acid, and 40 mg each of methyl, ethyl, propyl, and butyl parabens into 200 mL volumetric flask. Add ca. 50 mL 70% ethanol to dissolve, and dilute to volume with 70% ethanol.

For the standard curve, dilute 0.0, 1.0, 2.0, 3.0, 4.0, and 5.0 mL of mixed standard to 100 mL with 70% ethanol.

2. Storage Conditions

All standards are to be kept in stoppered glass bottles at 4° C.

3. Shelf Life Stability

Working standards: 6 months.

I. DETERMINATIVE METHOD

E. SAMPLE HANDLING

Sample Preparation

-
- a. Weigh 10 g thoroughly comminuted meat product or 2 g seasoning mixture into 250 mL Erlenmeyer flask. Select "blank" meat sample as control and for fortification.
 - b. Add 70 mL ethanol to sample in flask; add milli-Q water to make a final liquid volume of 100 mL. (Moisture content of meat product should be used in determining liquid volume.)
 - c. Break up lumps of meat with clean spatula. Add magnetic stirring bar, stopper, and mix on magnetic stirrer 10 min at medium speed.
 - d. Filter ca. 2 mL extract into 50 mL stoppered test tube using filter paper. (Refer to section B. 1, item c.)
 - e. Refilter ca. 2 mL aliquot of filtrate through 0.45 μ m filter into glass vial for LC analysis, using sample filtration apparatus. (Refer to section B. 1, item f.)
 - f. **Save remaining filtrate for GC/MS confirmatory analysis.**
 - g. With each set of official samples to be analyzed, process one reagent blank, one control, and one sample fortified with medium level (3.0 mL) mixed standard: 600 ppm benzoic acid, 24 ppm sorbic acid, and 60 ppm of each paraben.
-

I. DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE

Determination

-
- a. Linear gradient—Use linear gradient, programmed from 10–70% methanol in 1.5% aqueous ammonium acetate and 1.5% aqueous acetic acid over 10 min, with 10 min hold and 5 min equilibration delay at 1.5 mL/min. Equilibrate entire system with gradient elution of mobile phase until stable baseline is obtained (ca 30–45 min) at flow rate of 1.5 mL/min.
 - b. Using Wisp 710B sample processor, inject (in duplicate) 20 μ L portions each of sample extracts and mixed standards, and program solvent as described. Set range to 0.05 AUFS.

Under conditions used, retention times (min) of all 6 preservatives are as follows: benzoic acid, 5.80; sorbic acid, 7.05; methyl paraben, 8.23; ethyl paraben, 9.70; propyl paraben, 10.99; and butyl paraben, 12.14. (Refer to Figure 1 on facing page.)

- c. Calculate concentration of each preservative in sample as follows: Using peak areas or peak heights and concentrations of standards, construct linear standard curve for each compound based on formula $y = mx + b$, where x is peak area or height, y is concentration (ppm), m is slope, and b is the y intercept. Calculate recovery of fortified sample (included with every set), and correct sample results for recovery.
-

I. DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE (Continued)

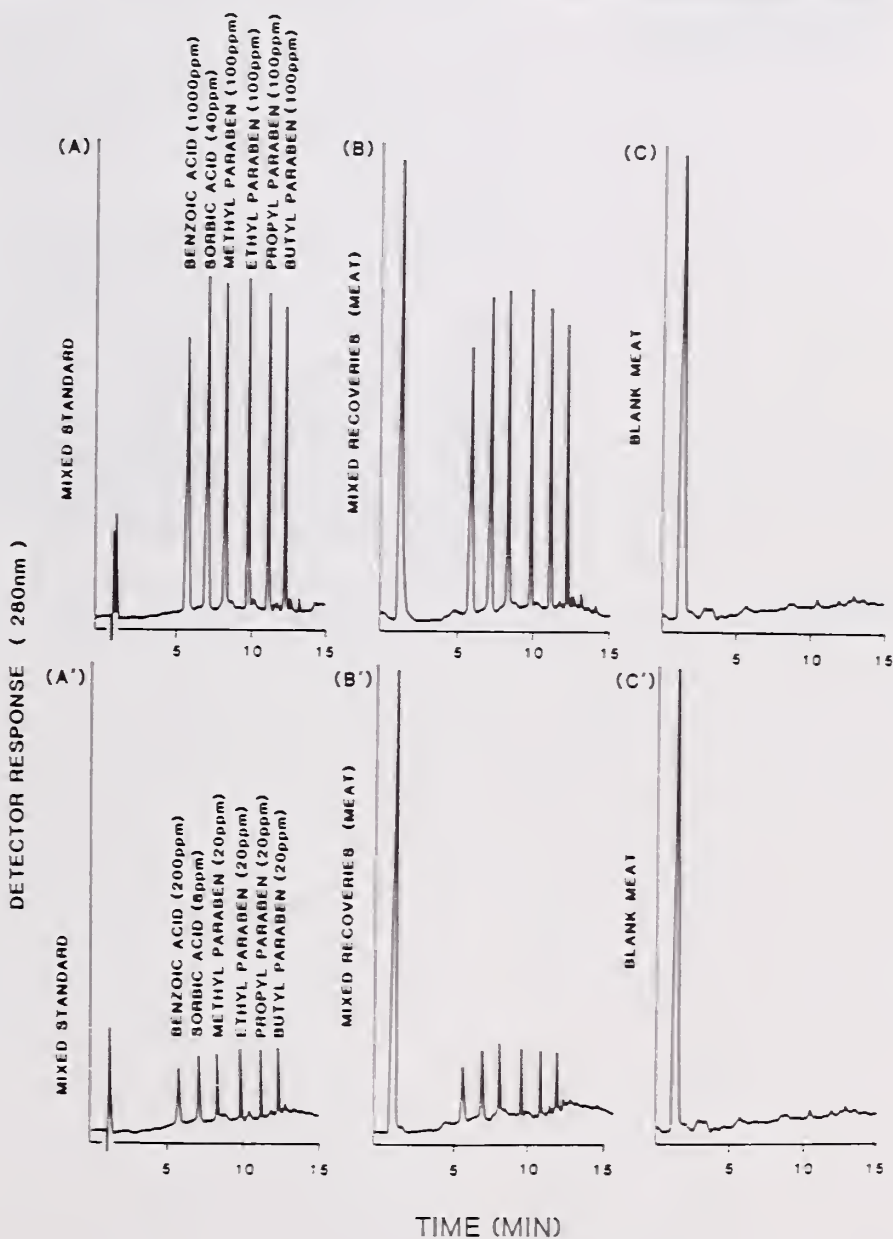


Figure 1. LC chromatograms of (A) mixed standards and (B) mixed recoveries of benzoic acid, sorbic acid, and 4 parabens at the highest levels used in the method and (C) unfortified meat: (A') and (B') mixed recoveries of benzoic acid, sorbic acid, and 4 parabens at the lowest level used in the method and (C') unfortified meat. UV detector, wavelength 280 nm, sensitivity 0.05 AUFS.

II. CONFIRMATORY METHOD

- | | |
|--|--|
| 1. Extraction of Benzoic Acid, Sorbic Acid, and Parabens for GC/MS Confirmation | <div style="border-top: 1px solid black; padding-top: 5px;"><ul style="list-style-type: none">a. Transfer remaining filtrate (refer to section E, Sample Preparation, step f) to 125 mL separatory funnel.b. Acidify with dilute HCl (1 + 1) to ca pH 3.0.<p style="margin: 5px 0;">NOTE: (Remainder of steps should be carried out in fume hood.)</p><ul style="list-style-type: none">c. Add 20 mL benzene, shake 1 min and allow phases to separate by letting stand 10 min.d. Discard lower aqueous layer and filter benzene layer through glass funnel containing ca. 5 g of anhydrous Na₂SO₄ into 25 mL test tube.e. Evaporate extract to dryness on evaporator at 60° C under nitrogen stream.f. Derivatize residue for GC/MS confirmation as follows: Inject residue in appropriate volume of acetone with constant time delay (TD), using N, O-bis (trimethylsilyl)-acetamide (BSA), into gas chromatograph equipped with either OV-3 or OV-7 liquid phase column for retention times of the molecular ions (M)⁺ and other important characteristic ions, e.g., (M-CH₃)⁺, (M-CH₃CO₂)⁺, (M-OSiMe₃)⁺, (M-COOSiMe₃)⁺. Also measure accurate masses of full mass spectra by comparison with reference standards.</div> |
| 2. Reference | <div style="border-top: 1px solid black; padding-top: 5px;"><p>Ali, M. Sher. <i>J. Assoc. Off. Anal. Chem.</i>, 1985, 68, 488-492.</p></div> |

CALCIUM/TRITIMETRIC

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DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory

Calcium is solubilized by acid hydrolysis forming calcium ion. The resultant hydrolyzate is diluted to a specific volume and an aliquot reacted with excess EDTA in alkaline media in the presence of cyanide and a hydroxy naphthol blue indicator. EDTA readily forms a chelated complex with the calcium ion. Excess EDTA is then titrated with calcium carbonate to a permanent purple end point. If phosphates are present they must be removed by passing an aliquot through an ion exchange column before the final titration steps.

2. Applicability

This procedure is applicable to the determination of calcium or bone in meat and poultry products.

DETERMINATIVE METHOD

B. EQUIPMENT

Apparatus

-
- a. Laboratory fume hood.
 - b. pH meter (Orion Model 701), or equivalent.
 - c. Volumetric labware: burets, flasks, pipets, etc.
 - d. Magnetic stirrer (Corning PC-353), or equivalent.
 - e. Hot plate.
 - f. Filtration funnel and filter paper (Whatman #4), or equivalent.
 - g. Glass beads.
 - h. Watch glass—about 80 mm diameter.
 - i. Chromatographic column—19 mm × 400 mm, fitted with a coarse-porosity scintered glass frit and teflon stopcock (Kontes #420540-0224), or equivalent.
 - j. 300 mL tall form beaker.
-

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

-
- a. 0.0200M EDTA (Disodium dihydrogen ethylene-diamine tetraacetic acid dihydrate: Dissolve 7.44 g $\text{Na}_2\text{H}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ (99 + % purity) in H_2O in 1 L volumetric flask, dilute to volume with H_2O , and mix. Weigh accurately 3 separate amounts of ACS primary standard CaCO_3 (about 80 mg) to give about 40 mL titration with 0.02M EDTA and transfer to 3 separate 500 mL Erlenmeyer flasks. To each, add 50 mL H_2O and enough HCl (1 + 3) to dissolve the CaCO_3 . Dilute each to about 150 mL with H_2O and add 15 mL 1N NaOH disregarding any ppt or turbidity. Add about 200 mg hydroxy naphthol blue indicator and titrate with EDTA solution from pink to deep blue end point, using magnetic stirrer. Add last few mL EDTA solution dropwise.

$$\text{Molarity EDTA solution} = \frac{\text{mg CaCO}_3}{\text{mL EDTA} \times 100.09}$$

Use the average molarity value of the three determinations.

- b. Hydroxy naphthol blue indicator (Mallinckrodt 5630).
- c. Potassium hydroxide-cyanide solution: Dissolve 280 g KOH in 500 mL water. Cool to room temperature, add 66 g KCN, dissolve, and dilute to 1 L. (Use care in handling KCN, as HCN is formed upon contact with water and acids.)
- d. HCl (1 + 1) and (1 + 3).
- e. NaOH (1 + 5) and 1N.

The following reagents are required only if phosphates are present:

- f. Amberlite IRA-93 resin (Rohm and Haas).
- g. 5% (w/v) sodium carbonate.
- h. HCl (3 + 22).
- i. 10% KOH (w/v in water).
- j. Phenolphthalein indicator: 1% (w/v) in ethanol.
-

DETERMINATIVE METHOD

D. STANDARDS

Preparation

0.02M CaCO_3 (ACS Primary Standard): Weigh 2.000 g CaCO_3 (dried at 100°C for 2 hr) into 1 L volumetric flask. Add 500 mL distilled water and 10-12 mL HCl (1 + 1). Heat just to boiling to dissolve. Dilute to volume with distilled water. Determine the relative strength ratio of the standardized EDTA solution to the CaCO_3 solution just before titrating samples (refer to section C, item a) as follows: Pipet 3 separate 25.0 mL portions of EDTA solution into separate 250 mL volumetric flasks. Dilute each to about 100 mL with H_2O and add 15 mL 1N NaOH, disregarding any ppt or turbidity. Add about 200 mg hydroxy naphthol blue indicator and titrate with the CaCO_3 solution from deep blue to pink end point, using magnetic stirrer. Add last few mL CaCO_3 solution dropwise.

$$\text{EDTA to CaCO}_3 \text{ ratio} = \frac{25.0 \text{ mL EDTA}}{\text{mL CaCO}_3 \text{ titrated}}$$

Use the average ratio from the three titrations.

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE

1. Determination

- a. Weigh 10.0 g sample into a 300 mL tall form beaker.
- b. Add 30 mL HCl (1 + 1), several glass beads, cover with watch glass, and place on hot plate in a fume hood.
- c. Slowly bring to a boil and digest for about 20 min.
- d. Cool to room temperature, filter into 200 mL volumetric flask. Wash filter paper with water until 200 mL filtrate is obtained, stopper, and mix.

At this point, if phosphates are present, proceed with removal outlined in section F.2.
- e. Pipette 20 mL aliquot into 400 mL beaker, add about 50 mL water. (Use 10 mL aliquot for samples containing greater than 0.85 percent calcium.)
- f. On a magnetic stirrer in a fume hood, add 200-300 mg hydroxy naphthol blue indicator, and adjust the pH to 12.5 ± 0.2 with KOH-KCN solution. (If pH exceeds 12.7, go back to step e, as $\text{Ca}(\text{OH})_2$ will be precipitated and it is insoluble.)
- g. Add 10-25 mL 0.02M EDTA (Amount depends on amount of calcium present. Must be in excess by at least 3 mL. Color should be green.) Mix on magnetic stirrer.
- h. Titrate with 0.02M CaCO_3 to a permanent purple end point.

2. Removal of Phosphates

- (Required only if product has been dipped, soaked, or injected with or in phosphate solutions.)
- a. Initial and regeneration of resin—Mix, in a beaker, approximately 35 g amberlite IRA-93 resin with three 250 mL portions of 5% sodium carbonate.
 - b. Wash with distilled water until washings indicate by phenolphthalein the absence of base.
 - c. Treat resin with three 250 mL portions of HCl (3 + 22), mixing thoroughly after each treatment.
 - d. Rinse with water until color is removed; transfer to chromatographic column with water. (The column is ready for use after water has drained to top of resin. The exchange capacity for phosphate is about 1,500 mg, so a number of aliquots can be passed through the column before regeneration is necessary. Before each use, rinse column with about 250 mL of water until elute is colorless.)
 - e. Transfer exactly 100 mL of sample solution from section F.1.d to a beaker. Adjust pH to 3.5 with 10% KOH, added drop by drop, using a pH meter and a magnetic stirrer.

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE (Continued)

-
- f. Pass entire solution through the resin column, at a rate of 2-3 mL/min, into a 250 mL volumetric flask.
 - g. Wash the beaker and column into the volumetric flask by passing through two 50 mL portions of water, the first at 2-3 mL/min, the second at 6-7 mL/min. Finally, freely pass enough water through column to get 250 mL total eluate. Stopper and mix.
 - h. Pipet 50 mL aliquot into 400 mL beaker and proceed as in section F.1.f-h.
-

DETERMINATIVE METHOD

G. CALCULATIONS

1. Procedure

$$\% \text{ Calcium content} = C = [A - (B \times R)](0.08) \left(\frac{M}{0.0200} \right)$$

Note: If 10 mL aliquot was used, multiply by 2 to obtain percent calcium content.

$$\% \text{ Bone for poultry} = (C - 0.015)F$$

where

A = mL 0.02M EDTA

B = mL 0.02M CaCO_3

0.015 = correction for natural calcium in poultry tissue

F = 6.25 for young chickens

= 4.55 for turkeys and mature chickens

R = EDTA to CaCO_3 ratio (from standardization)

M = molarity of EDTA

When analyzing mechanically deboned poultry that includes product from different age groups, calculate the bone multiplier as follows: (% of young chicken)(6.25) + (% of mature chicken)(4.55).

$$\text{Bone content of "conventionally" cooked poultry} = \frac{[(C) - 0.015](F)}{1.4}$$

NOTE: Raw deboned poultry contains approximately 23% solids. Conventionally cooked poultry will result in a 30% shrink of the fresh product, yielding approximately 33% solids. The factor 1.4 equates the bone content of conventionally cooked poultry to that of raw deboned poultry.

If inspector designates % solids processed other than by "conventional"

$$\text{cooking methods, bone content of such products} = \frac{[(C) - 0.015](F)(23)}{\% \text{ solids}}$$

DETERMINATIVE METHOD

G. CALCULATIONS (Continued)

For nutritional analyses:

United States Recommended Daily Allowance (USRDA) = 1.0 g

$$\text{mg/serving} = \frac{[A - (B \times R)](0.8 \text{ mg})\left(\frac{M}{0.0200}\right)(f)}{Wt}$$

where

A = mL 0.02M EDTA

B = mL 0.02M CaCO₃

R = EDTA to CaCO₃ (from standardization)

f = serving size converted to appropriate dimensions
(i.e., ounces to grams, etc.)

Wt = weight of sample in aliquot taken

2. Reference

- a. Wilson and Co. Method WC-29R1, 11/17/64.
 - b. JAOAC, 49, 287 (1966).
 - c. JAOAC, 50, 195, 219 (1967).
 - d. Hart and Fisher, "Modern Food Analysis," Springer-Verlag, NY (1971).
-

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Calcium, Titrametric Determination.
2. Required Protective Equipment	Safety glasses, plastic gloves, and lab coat.

3. Procedure Steps

	<u>Hazards</u>	<u>Recommended Safe Procedures</u>
Preparation of KOH-KCN solution	Chemical burn and HCN formation.	Perform mixing with care in a fume hood.
Digestion	Chemical burn and respiratory distress.	Perform digestion in fume hood.
pH adjustment	HCN formation.	Perfrom in fume hood.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standard

<i>Compound</i>	<i>Analytical Range (%)</i>	<i>Acceptable Recovery %</i>	<i>Repeatability % CV</i>	<i>Reproducibility % CV</i>
Calcium	0.03-0.85†	98-102	± 0.016‡	± 0.022‡

† 3-85 mg calcium in a 10 g sample.

‡ Standard deviation.

2. Critical Control Points and Specifications

<i>Record</i>	<i>Acceptable Control</i>
EDTA	EDTA 99% pure—0.200 ± 0.0005M
CaCO ₃	Primary standard—0.200 ± 0.0005M
EDTA/CaCO ₃	1:1 (NOTE: The actual ratio must be known within 0.001.)
End point	Color must be purple and persist for at least 1 min.
pH adjustment	pH 12.5 ± 0.2. If solution exceeds 12.7, analyst <i>must</i> go back to step F.1.e.
Phosphates removal (only required if carcasses have been soaked in phosphate solutions).	Pass through column—Amberlite IRA-93.
Calculations	Recheck.

3. Readiness To Perform

- a. Familiarization.
 - i. Phase I: Prepare standards, perform standardization.
 - ii. Phase II: Ten samples replicated on two different days, each analyst.
 - iii. Phase III: Check samples for analyst accreditation.
- b. Acceptability criteria.
See section J.1 above.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

4. Intralaboratory Check Samples

-
- a. System, minimum contents.
 - i. Frequency: Initially, minimum of 1 check sample biweekly per analyst.
 - ii. Blind samples or random replicates chosen by supervisor or Laboratory QA Officer after initial analysis.
 - iii. Records are to be maintained by analyst and reviewed by the supervisor and Laboratory QA Officer.
 - b. Acceptability criteria.

If unacceptable values are obtained, then:

 - i. Stop all official analyses for that analyst.
 - ii. Investigate and identify probable cause.
 - iii. Take corrective action.
 - iv. Repeat Phase III of section J.3 if cause was analyst-related.
-

5. Sample Acceptability and Stability

- a. Matrices—Mechanically separated (species)—MS(S); mechanically separated (kind)—MS(K).
 - b. Sample receipt size, minimum: Varied; enough to obtain matrix for all required quantitative results.
 - c. Condition upon receipt: Not spoiled or rancid, not leaking.
 - d. Sample storage:
 - i. Time: Indefinite.
 - ii. Condition: Frozen.
-

6. Sensitivity

-
- a. Lowest detectable level (LDL): NA.
 - b. Lowest reliable quantitation (LRQ): 0.03%.
 - c. Minimum proficiency level (MPL): 0.03%.
-

CHOLESTEROL IN MECHANICALLY SEPARATED SPECIES

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DETERMINATIVE METHOD

A. INTRODUCTION

Theory

The tissue is extracted with CHCl_3 -methanol. The extracted lipids are saponified with KOH and the unsaponifiable fraction is extracted and may or may not be derivatized for either GLC or HPLC quantitation.

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

-
- a. Tekmar SDT Tissuemizer, or equivalent.
 - b. 500 mL polypropylene centrifuge bottles.
 - c. Disposable microfilters (MSI Cameo Fisher Scientific Cat #DDN0400309).
 - d. 10 mL syringes, plastic disposable.
 - e. Buchner funnel.
 - f. 1 L suction flasks.
 - g. Whatman No. 1 filter paper.
 - h. 500 mL graduated cylinders.
 - i. Glass wool.
 - j. Glass funnels.
 - k. 300 mL Erlenmeyer flasks.
 - l. Steam bath.
 - m. Magnetic stirrer hot plate.
 - n. Glass column condensers.
 - o. Magnetic stirring bars.
 - p. 250 mL separatory funnels.
 - q. 100 mL round-bottom glass flask.
 - r. Rotary evaporator.
-

2. Instrumentation

-
- a. Gas chromatograph equipped with flame ionization detector, HP 5990, or equivalent, 6' \times 2 mm glass column packed with 3% OV17 on 100/120 Gas Chrom Q.
 - b. Isocratic HPLC system with 3.9 mm \times 30 cm, μ -Bondapak C₁₈ column and variable wavelength UV detector.
-

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and
Solution List

-
- a. Chloroform—Distilled in glass, Burdick and Jackson, or equivalent.
 - b. n-Heptane—Distilled in glass, Burdick and Jackson, or equivalent.
 - c. Acetonitrile—HPLC grade.
 - d. Ethyl ether—ACS reagent grade.
 - e. Petroleum ether—Distilled in glass, Burdick and Jackson, or equivalent.
 - f. Sodium sulfate (Na_2SO_4)—ACS reagent grade.
 - g. Potassium hydroxide (KOH) reagent ACS—50%, 1 N, and 0.5 N.
 - h. Reagent alcohol—Ethanol: Methanol: Isopropanol (90:5:5).
 - i. Celite 545.
 - j. Hexamethyldisilazane (HMDS)—No. 18006, Applied Science Laboratories, or equivalent.
 - k. Trimethylchlorosilane (TMCS)—No. 18010, Applied Science Laboratories, or equivalent.
 - l. Dimethyl dichlorosilane (DMCS)—No. 18008, Applied Science Laboratories, or equivalent.
 - m. Dimethylformamide (DMF)—Burdick and Jackson, or equivalent.
-

DETERMINATIVE METHOD

D. STANDARDS

**Preparation of
Standards**

-
- a. Cholesterol—(>99% purity) #C8667, Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178, or equivalent. Stock solution—1.0 mg cholesterol/mL chloroform. Dilute stock solution to obtain working standards of 0.1 mg/mL, 0.2 mg/mL, and 0.3 mg/mL in chloroform.
 - b. 5 alpha cholestane—#19505, Sigma Chemical Co. Stock solution—1.0 mg/mL n-heptane. Dilute stock solution with n-heptane for concentration of 0.2 mg/mL.
-

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE

1. Determination

(Perform moisture and fat analyses, MOI and FAT1 or FAT2.)

- a. Weigh quantity of sample to contain 0.5 to 1 g fat.
- b. Transfer to homogenizer container with 100.0 mL methanol.
- c. Add quantity of water to bring total water content to 40.0 mL.
- d. Add 50.0 mL CHCl_3 (NOTE: Ratio of CHCl_3 :methanol:water must be 50:100:40) and blend 3 min at high speed.
- e. Add 50.0 mL CHCl_3 and blend 0.5 min at medium speed.
- f. Add 50.0 mL water and blend 0.5 min at medium speed.
- g. Filter homogenate under vacuum into 1L suction flask through Buchner funnel, fitted with Whatman No. 1 paper containing 2 g Celite 545. (Optional: Before filtration, centrifuge at 2500 rpm for 10 min to remove denatured protein, which may clog the filter.)
- h. Transfer filtrate into 500 mL graduated cylinder.
- i. Transfer filter and contents to homogenizer container and add 90 mL chloroform. Blend 0.5 min at medium speed. Filter chloroform extract without additional Celite under vacuum. (Optional: Before filtration, centrifuge at 2500 rpm for 5 min.)
- j. Transfer filtrate to graduated cylinder containing original filtrate.
- k. Wash mixer cup and filter cake with two 15 mL portions of CHCl_3 . Add to original filtrate and allow layers to separate (if emulsion develops, centrifuge filtrate 5 min at 2500 rpm).
- l. Record volume of CHCl_3 layer (should be ca 180-200 mL). Aspirate and discard aqueous-alcohol layer.
- m. Filter 100 mL aliquot chloroform extract through glass funnel containing a small pledget of glass wool and 25 g of anhydrous Na_2SO_4 into 150 mL beaker.
- n. Rinse Na_2SO_4 with 15 mL CHCl_3 . Evaporate under gentle stream of N_2 on water or steam bath.
- o. Dissolve residue in ca 70 mL petroleum ether and filter through Whatman No. 1 paper containing 20 g anhydrous Na_2SO_4 into 300 mL glass-stoppered Erlenmeyer flask.
- p. Rinse beaker and Na_2SO_4 with several 10 mL portions of petroleum ether. Evaporate under gentle steam of N_2 on water or steam bath.

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE (Continued)

-
- q. Introduce magnetic stirring bar and place on magnetic stirrer hot plate. With gentle stirring slowly add 10 mL 50% KOH and 40 mL reagent alcohol.
 - r. Attach condenser, turn on hot plate, and reflux 1 hr.
 - s. Turn off heat and add 60 mL reagent alcohol through condenser into saponified solution while stirring and cooling.
 - t. When solution ceases to reflux, remove condenser and pipet 100.0 mL ethyl ether into solution while stirring slowly. Remove stirring bar, stopper flask, and shake vigorously 30 sec.
 - u. Pour into 500 mL separatory funnel (do not rinse flask). Add 200 mL 1N KOH and shake vigorously 10 sec. Allow layer to separate and discard aqueous phase.
 - v. Add 40 mL 0.5N KOH, rotate gently end to end for 10 sec, allow layers to separate, and discard aqueous layer.
 - w. Pour ethyl ether layer into 250 mL separatory funnel. Add 40 mL distilled water and rotate separatory funnel end to end ten times. Allow layers to separate and discard aqueous layer. Repeat water wash 3-5 more times. The pH of the last water wash should be about 7.
 - x. Pour ethyl ether layer from top of the separatory funnel into 125 mL glass stoppered Erlenmeyer flask containing 30 g anhydrous Na_2SO_4 . Stopper and shake flask vigorously. Let stand 15 min.
-

2. Chromatographic Conditions

- a. GLC—Carrier gas: nitrogen, 30-40 mL/min.
Flame: Hydrogen/air; 1/10 (approximate, adjust for maximum sensitivity).
Oven temperature: 250° C.
Detector temperature: 300° C.
Injection port temperature: 300° C.
 - b. HPLC—Mobile phase: 100% acetonitrile.
Flow rate: 1.0-2.0 mL/min (optimize for instrument).
Detector: 206 nm, 0.01 AUFS.
Injector size: 10 μ l loop.
-

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE (Continued)

3. Standard Curve Preparation

GLC—To prepare standard curve use 0.10, 0.2, and 0.3 mg/mL standards in CHCl_3 . (This should bracket sample concentration: 0.5-1.0 g fat in 100 mL CHCl_3 .) Inject standards directly or derivatize.

a. Derivatization.

- i. Transfer 1.0 mL of each standard solution into a 15 mL silanized centrifuge tube.
- ii. Add 0.2 mL HMDS and 0.1 mL TMCS.
- iii. Stopper tube and shake vigorously (either by hand or on mechanical vibrator) for 30 sec.
- iv. Let solution stand for 15 min.
- v. Add 1.0 mL 5 alpha-cholestane internal standard (0.2 mg/mL) and 10 mL distilled water to tube.
- vi. Vigorously shake 1 min and then centrifuge 2 min.
- vii. Inject 3 μl of each standard or other appropriate volume of heptane layer into gas chromatograph.
- viii. Determine area of each peak by using height-widths measurement or digital integrator.
- ix. Divide cholesterol peak area by internal standard peak area to obtain standard response ratio. Obtain response factor by dividing response ratio by weight of standard (mg/mL standard.) If standard response factors are within 4% of each other, use average response factor to calculate cholesterol content of sample. If the response factors are not within 4% of each other, new standards should be prepared and/or the gas chromatography parameters should be reviewed.

b. HPLC—Direct.

Inject appropriate volumes of the 0, 0.1 mg/mL, 0.2 mg/mL, 0.3 mg/mL in CHCl_3 standards (this should bracket sample concentration: 0.5-1.0 g in 100 mL CHCl_3) into HPLC. Plot standard curve.

4. Derivatization and Quantitation

a. GLC (derivatization).

- i. Pipet 50 mL ethyl ether layer from step F.1.x into 100 mL round-bottom glass stoppered flask and roto-evaporate to dryness at 40° C. Add 3 mL acetone and evaporate to dryness. Dissolve residue in 3.0 mL DMF.
- ii. Transfer 1.0 mL residue solution to 15 mL silanized centrifuge tube and derivatize as in derivatization for cholesterol standard curve beginning at step F.3.a.ii.

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE (Continued)

-
- iii. Inject 3 μ l or other appropriate volume. Determine areas of cholesterol and internal standard peaks. $\text{Mg cholesterol/100 g sample} = (\text{peak area of cholesterol/peak area of internal standard} \times 100)/\text{response factor} \times \text{g sample used for derivatization}$.
- b. GLC (direct).
 - i. Transfer 50 mL ethyl ether layer from step F.1.x into 100 mL round-bottom flask. Wash Na_2SO_4 two times with 10 mL ethyl ether. Add washes to flask. Roto-evaporate to dryness at 40° C.
 - ii. Transfer residue quantitatively with CHCl_3 into 25 mL volumetric flask. Make to volume with CHCl_3 and mix well.
 - iii. Inject appropriate volume into GLC. Measure peak height and determine cholesterol from standard curve prepared as in section F.3.
 - c. HPLC (direct).
 - i. Transfer 50 mL ethyl ether layer from step F.1.x into 100 mL round-bottom glass-stoppered flask and roto-evaporate to dryness at 40° C. Add 3 mL acetone and roto-evaporate to dryness.
 - ii. Transfer residue with CHCl_3 into 25 mL volumetric flask, make to volume with CHCl_3 , and mix well.
 - iii. Inject appropriate volume into HPLC and calculate cholesterol from standard curve prepared as in section F.3.
-

5. References

-
- a. Punwar, J. K., "Gas-Liquid Chromatographic Determination of Total Cholesterol in Multi-component Foods," *JAOAC*. 58, 1975, 804-810.
 - b. Punwar, J. K., "Collaborative Study for the Comparison of Two Methods for the Determination of Total Cholesterol in Multicomponent Foods," *JAOAC*. 59, 1976, 46-50.
 - c. Sheppard, A. J. et al., "Gas-Liquid Determination of Cholesterol and Other Sterols in Foods," *JAOAC*. 60, 1977, 1302-1306.
 - d. Newkirk, D.R. and Sheppard, A. J., "High-Pressure Liquid Chromatographic Determination of Cholesterol in Foods," *JAOAC*. 64, 1981, 54-57.
-

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standard

<i>Compound</i>	<i>Analytical Range (ng)</i>	<i>Acceptable Recovery %</i>	<i>Repeatability % CV</i>
Cholesterol	> 10	80 – 110	< 15.0

2. Critical Control Points and Specifications

<i>Record</i>	<i>Acceptable Control</i>
Extraction temperature	68° – 80° F

3. Readiness To Perform

a. Familiarization.

i. Phase I: Standards—5 levels, 3 replicates each

- 100 µg/mL
- 200 µg/mL
- 300 µg/mL
- 400 µg/mL
- 500 µg/mL

ii. Phase II: Fortified samples—(Tissue blanks must be analyzed to determine naturally occurring cholesterol—analyze unfortified sample 5 times—CV < 10%) Add nominal levels to produce 100, 200, 300, 400, and 500 µg/mL final solutions; 3 each.

Submit data from standards and fortified samples to Chemistry Division for information.

NOTE: Phases I and II may be performed concurrently.

iii. Phase III: Check samples for analyst accreditation.

- 5 samples from FSIS Western Laboratory (Samples submitted by supervisor if only one laboratory is performing this test.)
- Report analytical findings to Chemistry Division.

Notification from Chemistry Division required to commence official analysis.

b. Acceptability criteria.

Recoveries

- 80 – 110%, range of individual recoveries
- Coefficient of variation of < 15% for recoveries
- Coefficient of variation of < 10% for mean ratios

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

4. Intralaboratory Check Samples

a. System, minimum contents.

- i. Frequency—1 per week per analyst, or 20% of official samples analyzed (whichever is smaller).
- ii. Blind samples (requires "dummy" forms).
- iii. Records are to be maintained by analyst and reviewed by the supervisor and Laboratory QA Officer.
 - All replicate findings
 - Running average difference between replicates
 - All % recoveries
 - Running average, standard deviation, and CV for recoveries
 - Appropriate CUSUM charts

b. Acceptability criteria.

If unacceptable values are obtained then:

- i. Stop all official analyses for that analyst.
 - ii. Investigate and identify probable cause.
 - iii. Take corrective action.
 - iv. Repeat Phase III of section J.3 if cause was analyst-related.
-

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

6. Sample Acceptability and Stability

-
- a. Matrices of choice—Any meat or meat product requiring cholesterol analysis.
 - b. Sample receipt size—Varied; enough to produce matrix required for all quantitative tests.
 - c. Condition upon receipt—Hard frozen or ice crystals present.
 - d. Sample storage:
 - i. Time—Indefinite.
 - ii. Condition—Frozen.
-

7. Sensitivity

-
- a. Lowest detectable level (LDL):
 - b. Lowest reliable quantitation (LRQ):
 - c. Minimum proficiency level (MPL): > 10 ng.
-

CEREAL

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DETERMINATIVE METHOD

A. INTRODUCTION

Theory

Cereal is added to meat food products as a binder. In this procedure, the cereal starch is dissolved in 1 + 1 HCl, precipitated, and determined gravimetrically. A rapid semiquantitative method is also described.

As in the SOY1 and SOY2 methods, the meat is rendered soluble by treatment with an alcoholic solution of caustic potash, spices and cereal starch remaining as a sediment.

If a semiquantitative estimation of the cereal content is desired, this residue volume is read and a deduction allowed for spices.

A more accurate quantitation is obtained if the cereal starch is dissolved in 1 + 1 HCl, precipitated with 95% ethanol, dried, and weighed.

DETERMINATIVE METHOD

B. EQUIPMENT

Apparatus

-
- a. Centrifuge, with a 6-7/8" diameter head.
 - b. Centrifuge tubes, Goetz, 100 mL Corning, Catalog #8220, or equivalent.
 - c. Gooch crucible.
 - d. Whatman filter paper 541, or equivalent.
-

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List
a. Ethanol, 95%.
b. Alcoholic caustic potash solution (8%): Dissolve 40 g of KOH in 300 mL of 95% ethanol and dilute to 500 mL with 95% ethanol.
c. Dilute hydrochloric acid (1 + 1): Mix 1 vol of concentrated HCl with 1 vol of distilled H ₂ O.
d. Dilute hydrochloric acid (1:3): Mix 1 vol of concentrated HCl with 3 vol of distilled H ₂ O.

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE

1. Determination— Semiquantitative

(Applicable only in the absence of soybean flour and soy protein concentrate.)

- a. Weigh 10.0 grams of sample into a 100 mL Goetz tube.
 - b. If corn syrup, corn syrup solids, nonfat dry milk, and/or calcium reduced dry skim milk are present, extract with two successive 50 mL portions of warm, distilled H₂O; shake, centrifuge, decant, and discard the supernatant liquid after each extraction. (If CS, CSS, NFDM, or CRDSM are absent, this extraction is to be omitted.)
 - c. Add 50 mL of 8% alcoholic KOH solution, digest on steam bath for 20 min with occasional stirring, then dilute to 100 mL with 95% ethanol.
 - d. Allow to stand for 1 hr and read volume of sediment in tube.
-

2. Determination— Gravimetric

- a. Centrifuge the 100 mL suspension for 5 min. Decant and discard the supernatant liquid.
 - b. Wash the residue with 25 mL of 95% ethanol, stirring the sediment thoroughly.
 - c. Centrifuge, decant, and discard the supernatant liquid.
 - d. If soya flour or concentrate is present, add 50 mL of 1 + 3 HCl, mix thoroughly, stopper, and shake for 1 min. Centrifuge at 2000 rpm for 4 min, decant, and discard supernatant. (If soya is absent, skip this step.)
 - e. Add 50 mL of 1 + 1 HCl, mix thoroughly, stopper, and shake for 1 min.
 - f. Centrifuge at 2000 rpm for 4 min. If the supernatant liquid is not clear, filter it through a double thickness 541 Whatman paper, or equivalent.
 - g. Transfer 25 mL of clear supernatant liquid to a 150 mL beaker containing 75 mL of 95% ethanol, mix well, and let stand for 1 hr.
 - h. Filter through a tared Gooch crucible, wash with two 25 mL portions of 95% ethanol, dry for 30 min at 75° C, and weigh.
-

DETERMINATIVE METHOD

G. CALCULATIONS

1. Procedure

-
- a. Semiquantitative determination.

% cereal = Volume of sediment in tube—0.5% for spices, if present.

- b. Gravimetric determination.

$$\% \text{ cereal} = \frac{(A - B)(1.45)(100)}{\frac{C}{2}}$$

A = Weight of Gooch crucible + starch

B = Weight of Gooch crucible

C = Sample weight

1.45 = Factor for converting from starch to cereal, assuming that cereals contain an average starch content of 69%.

2. References

-
- a. Official Methods of Analysis of the Association of Official Analytical Chemists, 14th Edition (semiquantitative only).
- b. Chemistry Laboratory Guidebook, 1971 (gravimetric only).
-

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Cereal Determination.		
2. Required Protective Equipment	Safety glasses, plastic gloves, and lab coat.		
3. Procedure Steps		<u>Hazards</u>	<u>Recommended Safe Procedures</u>
	C. Reagents		
	Determination— Semiquantitative (c) Add 50 mL of 8% alcoholic KOH solution—etc.	Skin and eye irritation	Procedure should be performed in an efficient fume hood.
	Determination— Gravimetric (b) Wash the residue with 95% ethanol,—etc. (d) Add 50 mL 1 + 1 HCl, etc.	Dermal and respiratory irritation	In addition to using an efficient fume hood, it would be desirable to locate the centrifuge within arms reach of the hood.
4. Disposal Procedures	Alcoholic KOH and sample digest solution	Skin and eye irritation.	Flush into disposal sink with large quantities of water.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standard

<i>Compound</i>	<i>Analytical Range % percent</i>	<i>Repeatability CV %</i>	<i>Reproducibility CV %</i>
Cereal	‡	<15.0	<20

‡ Limit may vary due to sample and aliquot sizes and sample type.

2. Critical Control Points and Specifications

<i>Record</i>	<i>Acceptable Control</i>
a. <i>Semiquantitative:</i>	
Sample size	10 g \pm 0.1 g.
MP form check	Corn syrup, corn syrup solids, nonfat dry milk, calcium-reduced nonfat dry milk: if present, must be removed by extraction step.
Calculation	Recheck.
b. <i>Quantitative:</i>	
Sample size	10 g \pm 0.1 g
MP form check	Same as above.
Dilute hydrochloric acid	Must be 1 + 1.
Aliquot	25 mL with pipet.
Standing time	1 hr \pm 5 min.
Drying temperature and time	75° C \pm 2° C for 30 min \pm 5 min.
Calculation	Recheck.

3. Readiness To Perform

a. Familiarization.
i. Phase I: Standards—NA.
ii. Phase II: Fortified samples
iii. Phase III: Check samples for analyst accreditation.
b. Acceptability criteria.
See section J.1 above.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

4. Intralaboratory Check Samples

-
- a. System, minimum contents.
 - i. Frequency: As determined by the supervisor.
 - ii. Blind samples or random replicates chosen by supervisor after initial analysis.
 - iii. Records are to be maintained by analyst and reviewed by the supervisor and Laboratory QA Officer.
 - b. Acceptability criteria.

If unacceptable values are obtained, then:

 - i. Stop all official analyses for that analyst.
 - ii. Investigate and identify probable cause.
 - iii. Take corrective action.
 - iv. Repeat Phase III of section J.3 if cause was analyst-related.
-

5. Sample Acceptability and Stability

-
- a. Matrix: meat and meat product
 - b. Sample receipt size, minimum: 1 lb.
 - c. Condition upon receipt: 4° C and sealed from atmosphere.
 - d. Sample storage:
 - i. Time: One month.
 - ii. Condition: 4° C.
-

6. Sensitivity

-
- a. Lowest detectable level (LDL): undetermined
 - b. Lowest reliable quantitation (LRQ): 0.3%
 - c. Minimum proficiency level (MPL): 0.3%.
-

CORN SYRUP SOLIDS

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DETERMINATIVE METHOD

A. INTRODUCTION

Theory

Corn syrup solids (CSS) are added to meat food products as a flavoring agent. In this procedure, the amount of added CSS is determined by analyzing the product for its maltose content.

Because recent studies have indicated that the maltose content of CSS is quite variable, it is imperative that the same lot of CSS be analyzed for this constituent; this value is then used in calculating the CSS content of the product.

If corn syrup (CS) is used, a sample of the CS should be analyzed for moisture content, and only those samples that assay 20% or less moisture should be permitted in the product.

Samples to which CSS (or CS) and nonfat dry milk (NFDM) or calcium-reduced dry skim milk (CRDSM) have been added, are analyzed for CSS content by determining the difference between the reducing sugars remaining in the sample after it has been subjected to two fermentations: (1) by washed yeast (which leaves lactose and maltose), and (2) by yeast acclimated to maltose (which leaves lactose).

Samples to which CSS or CS, but neither NFDM or CRDSM, have been added, are analyzed for CSS content by determining the amount of maltose present following a washed yeast fermentation.

NOTE: A substantial portion of section A.1 of the NFDM1 method is applicable to this method.

DETERMINATIVE METHOD

B. EQUIPMENT

Apparatus

All apparatus may be substituted with an equivalent.

- a. Centrifuge—Model C-6000 (IEC 3504).
 - b. Swinging Bucket Rotor—IEC 256
 - c. Central adapter for 250 mL tubes—IEC 5780.
 - d. Centrifuge tubes—250 mL conical bottom with cap Fisher #05-538-53.
 - e. Cushion for 05-538-53 tubes—Fisher #05-538-53A.
 - f. Incubator—6000 series standard lab incubator—Fisher #11-683-655D.
 - g. Precision controlled rheostat heater—Fisher #11-425.
 - h. Standard laboratory glassware.
-

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

**Reagent and
Solution List**

-
- a. Washed yeast suspension.
- Mix four cakes of baker's yeast (or 30 g of active dry yeast) to a smooth suspension with 300 mL of distilled H₂O (if active dry yeast is used, the yeast must be added to the water).
 - Centrifuge for 5 min and discard aqueous layer. Repeat four more times, or until supernatant is clear following centrifugation.
 - Suspend by stirring the yeast in distilled H₂O, dilute to 200 mL with distilled H₂O, and refrigerate at about 4° C.
- b. Acclimated yeast suspension.
- Prepare acclimating medium by dissolving each of the following ingredients in a small amount of distilled H₂O and adding, in the order given, to 1,000 mL of distilled H₂O.
 - 2.0 g anhydrous MgSO₄.
 - 4.0 g NH₄Cl.
 - 2.0 g anhydrous K₂HPO₄.
 - 1.0 g KCl.
 - 0.04 g FeSO₄•7H₂O.
 - 1.4 g peptone.
 - 40.0 g technical maltose.
 - Dilute to 2 L, warm, and filter. Bring filtrate to a rolling boil and cool to room temperature.
 - Shake well the washed yeast suspension obtained in a; remove 100 mL and centrifuge.
 - Discard the aqueous layer, add the washed yeast to 1 L of the acclimating medium, and incubate for approximately 24 hr at 30° C, stirring frequently the first few hours.
 - Separate yeast by decanting and centrifuging. Wash twice with distilled H₂O and repeat incubation with the remaining 1 L of acclimating medium.
 - Separate yeast again, wash 4 or 5 times with distilled H₂O, suspend yeast in distilled H₂O, dilute to 100 mL with distilled H₂O, and refrigerate at about 4° C.

NOTE: To determine the viability and potency of the acclimated yeast suspension: Weigh 500 mg of maltose and 800 mg of dextrose and transfer to a 100 mL volumetric flask. Dilute to volume with distilled H₂O, stopper, and mix well. Pipet a 10 mL aliquot into a 50 mL volumetric flask and

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS (Continued)

proceed through the incubation procedure. Boiling 10 mL of this solution (following centrifugation) with 20 mL of Benedict's solution for exactly 3 min should yield no precipitate or suspension of Cu_2O , indicating that the yeast fermented the sugars. If a precipitate or suspension occurs, the yeast should be discarded. This is the easiest procedure for determining that the yeast is "working" properly. [If testing washed yeast, weigh only 800 mg of dextrose. Do not use any maltose.]

- c. Dilute hydrochloric acid (1 + 4): One volume conc. HCl + 4 volumes distilled H_2O .
 - d. Phosphotungstic acid: 20% w/v.
 - e. Chlorophenol red indicator: Dissolve 0.1 g chlorophenol red in 2.4 mL of 0.1N NaOH and dilute to 250 mL with distilled H_2O .
 - f. Bromthymol blue indicator: Dissolve 0.1 g bromthymol blue in 1.6 mL of 0.1N NaOH and dilute to 250 mL with distilled H_2O .
 - g. Buffer solution, pH 4.8.
 - i. Prepare 0.1M citric acid (19.21 g/L) and 0.2M Na_2HPO_4 (28.4 g anhydrous/L).
 - ii. Mix solutions in proportions of 10.14 mL citric acid to 9.86 mL Na_2HPO_4 and adjust to pH 4.8, using a pH meter.
 - iii. Store in refrigerator and discard if solution becomes turbid.
 - h. Benedict solution.
 - i. Dissolve 16 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 150 mL of distilled H_2O .
 - ii. Dissolve 150 g sodium citrate dihydrate, 130 g anhydrous Na_2CO_3 , and 10 g NaHCO_3 in 650 mL of distilled H_2O .
 - iii. Combine the two solutions, cool, dilute to 1 L with distilled H_2O , and filter.
 - i. Dilute acetic acid: Dilute 240 mL glacial acetic acid to 1 L with distilled H_2O .
 - j. Dilute phosphoric acid: Dilute 240 mL phosphoric acid to 1 L with distilled H_2O .
 - k. Iodine standard solution: Dissolve 10.2 g KI in minimum quantity of distilled H_2O and use this solution as a solvent for 5.08 g I_2 . Filter, if necessary, through a glass fiber filter paper and dilute to 1 L with distilled H_2O .
 - l. Sodium thiosulfate standard solution: Dissolve 9.92 g $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ in recently boiled, cooled distilled H_2O and 0.1 g Na_2CO_3 , and dilute to 1 L with distilled H_2O .
 - m. Starch indicator solution: Triturate 2 g of soluble starch and 10 mg HgI_2 with a small amount of distilled H_2O . Add the suspension slowly to 500 mL boiling distilled H_2O and boil until clear.
-

DETERMINATIVE METHOD

D. STANDARDS

Preparation of Standards

-
- a. Maltose standard solution: Dissolve 1.5789 g maltose monohydrate in distilled H₂O and dilute to 1 L with distilled H₂O (10 mL = 15 mg anhydrous maltose).
 - b. Dextrose standard solution: Dissolve 1.500 g dextrose in distilled H₂O and dilute to 1 L with distilled H₂O (10 mL = 15 mg dextrose).
-

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE

1. Determination

- a. Weigh 20.0 sample into a 200 mL volumetric sugar flask.
- b. Add 50 mL distilled H₂O, stir or shake to break up any lumps, and heat on steam bath for 30 min.

NOTE: The small amount of water added to the 20 g sample should be at room temperature when added to the sugar flask. If it is not, coagulation of the meat and milk protein might occur, making it difficult to macerate the sample and leach out the maltose.

- c. Cool to room temperature, add 20 mL dilute HCl, and dilute to volume, using bottom of fat layer as meniscus.

NOTE: If following the 30-min heating time on the steam bath the flask and contents are not cooled to room temperature prior to adding the HCl, loss of maltose may take place by hydrolysis.

- d. Add 10 mL of 20% phosphotungstic acid solution, mix, let stand for a few minutes, and filter through a moistened filter paper.
- e. For samples containing both CSS (or CS) and NFDM (or CRDSM), pipet two 40 mL aliquots of protein-free filtrate into 50 mL volumetric flasks.
 - i. Neutralize one just to the acid side of the bromthymol blue indicator, dilute to volume with distilled H₂O, and mix.
 - ii. Neutralize the other just to the acid side of the chlorophenol red indicator, add 5 mL of the buffer solution, dilute to volume with distilled H₂O, and mix.
 - iii. To a centrifuge tube, add 5 mL of washed yeast suspension; to another centrifuge tube, add 5 mL of acclimated yeast suspension.
 - iv. Separate the H₂O by centrifuging and decanting.
 - v. Transfer about 40 mL of the unbuffered solution to the centrifuge tube containing the washed yeast.
 - vi. Transfer about 40 mL of the buffered solution to a centrifuge tube containing the acclimated yeast. Mix yeast and sample well; incubate washed yeast for 1 hr at 30° C and acclimated yeast for 3 hr at 30° C, stirring frequently.
 - vii. Centrifuge.
 - viii. Pipet 10 mL of clear supernatant into a 300 mL Erlenmeyer flask, add 20 mL of Benedict solution, cover with watch glass and bring to boil in 3-5 min, and boil for exactly 3 min.

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE (Continued)

-
- ix. Remove from heat, cool rapidly, and add 100 mL distilled H₂O and 10 mL dilute acetic acid slowly while swirling. Keep covered with watch glass.

NOTE: The reducing portion of this method is extremely critical because it involves an empirical procedure. The 3-min boiling time must be strictly adhered to, and the flask should be cooled rapidly following boiling; for example, invert a beaker over the neck of the flask and allow a stream of cold tap water to flow over the flask.

- x. Add a definite volume of standard iodine solution (15 mL for about 1.5% maltose, or 30% excess) and agitate to dissolve the Cu₂O. Keep covered with watch glass.
- xi. Allow flask to stand at least 5 min. With watch glass just ajar, add 20 mL of dilute phosphoric acid solution. Slowly swirl to mix. Keep watch glass on. Rinse underside of watch glass into flask with distilled water before titrating excess iodine with standard sodium thiosulfate solution, using starch as an indicator.

NOTE: The titration should be performed immediately after the addition of the H₃PO₄ to avoid any possible loss of I₂. The use of more costly iodine flasks will also serve to avoid loss of I₂.

- xii. Determine I₂:Na₂S₂O₃ ratio by using 10 mL of distilled water and carry through determination as above, beginning with step viii “. . . add 20 mL of Benedict solution . . .”

$$I_2:Na_2S_2O_3 \text{ ratio} = \frac{\text{Volume } I_2 \text{ (mL)}}{\text{mL } Na_2S_2O_3} = A$$

- xiii. Determine maltose:I₂ ratio by using 10 mL of standard maltose solution and carrying through determination as above, beginning with step viii “. . . add 20 mL of Benedict solution . . .”

$$\text{Maltose}:I_2 \text{ ratio} = \frac{15 \text{ mg maltose}}{\text{mL } I_2 - (\text{mL } Na_2S_2O_3)(A)} = B$$

- f. For samples containing either CSS or CS, but neither NFDM nor CRDSM, pipet a single 40 mL aliquot of protein-free filtrate into a 50 mL volumetric flask.
- i. Neutralize just to the acid side of the bromthymol blue indicator, dilute to volume with distilled H₂O, and mix.
- ii. Transfer about 40 mL of this solution to a centrifuge tube to which 5 mL of washed yeast suspension has been added and from which the H₂O has been separated.
- iii. Mix yeast and sample well and incubate for 1 hr at 30° C. Proceed as above, beginning with step e. vii.
-

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE (Continued)

2. Dextrose Determination

This procedure may also be used to determine total reducing substances (total sugars calculated as dextrose) in a sample by performing the method as above, except for the following changes.

- a. Add 20 mL dilute HCl at step 1.b rather than 1.c.
 - b. In step 1.e or 1.f, dilute filtrate to 50 mL.
 - c. Skip steps 1.e.i. through 1.e. vii. or 1.f.i. through 1.f.viii. and proceed as in 1.e.viii.
 - d. Determine the dextrose: I_2 ratio in step 1.e.xiii. by using the dextrose standard (section D, item b) instead of the maltose standard.
-

DETERMINATIVE METHOD

G. CALCULATIONS

1. Procedure

-
- a. For product containing CSS (or CS) and NFDM (or CRDSM)

$$\text{Percent maltose} = \frac{100 [(D - AE) - (F - AG)] [B]}{C}$$

- b. For product containing CSS or CS, but neither NFDM nor CRDSM

$$\text{Percent maltose} = \frac{100 [D - AE] [B]}{C}$$

A = I_2 : $Na_2S_2O_3$ ratio

B = Maltose: I_2 ratio

C = Milligrams of sample in aliquot (consider the volume of the original sample solution as 200 mL, rather than 210 mL, to take into account the volume occupied by the sample).

D = mL of I_2 added to flask (washed yeast)

E = mL of $Na_2S_2O_3$ required for back titration (washed yeast)

F = mL of I_2 added to flask (acclimated yeast)

G = mL of $Na_2S_2O_3$ required for back titration (acclimated yeast)

$$\text{Percent CSS} = (100) \frac{\text{Percent Maltose}}{\text{Percent Maltose in CSS}} - H$$

H = Correction factor to be applied when NFDM or CRDSM is absent = 0.4%

No correction factor should be applied when NFDM or CRDSM is present.

2. Reference

Chemistry Laboratory Guidebook, 1971.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Corn Syrup Solids Determination.		
2. Required Protective Equipment	Safety glasses, plastic gloves, lab coat.		
3. Procedure Steps			
		<u>Hazards</u>	<u>Recommended Safe Procedures</u>
	C. Reagents		
	Dilute hydrochloric acid Phosphotungstic acid Dilute acetic acid Dilute phosphoric acid	Skin, eye, and respiratory irritation.	All these bulk reagents should be prepared in well-ventilated areas and dispensed using repipettors wherever practical.
4. Disposal Procedures	Reagent waste	See above	Flush into waste disposal sink with large quantities of water.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standard

<i>Compound</i>	<i>Analytical Range (%)</i>	<i>Repeatability % CV</i>	<i>Reproducibility % CV</i>
Corn Syrup	‡	< 15.0	< 20

‡ Limit may vary due to sample and aliquot sizes and sample type.

2. Critical Control Points and Specifications

<i>Record</i>	<i>Acceptable Control</i>
Yeast preparations	Prepare <i>exactly</i> as written. Activity checked using control sample containing NFDM.
pH 4.8 buffer solution	Check on pH meter before use. Calibrate meter with pH 4 or 5 buffer.
Benedict's solution	All weights of constituents are critical to ± 0.1 g. Must be filtered before use.
Iodine and thiosulfate standard solutions	1:1 (ratio) ± 0.1 .
Maltose standard solution	1.5789 g maltose diluted to 1 L.
Sample size	20 g ± 0.1 g.
Steam bath	All sample lumps must be broken up.
After steam bath	Must be cooled to room temperature before addition of dilute HCl.
Filter paper	Must be moistened before filtering, after phosphotungstic acid addition.
Neutralization	Proper pH depending on presence or absence of corn syrup or solids.
Yeast	Washed in absence of corn syrup; acclimated in presence of corn syrup.
Incubation temperature	30° C ± 1 ° C.
Incubation time	1 hr ± 5 min: washed yeast; 3 hr ± 5 min: acclimated yeast.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

Boiling with Benedict's solution	Cover with watch glass. Bring to boil in 4 ± 1 min. Boil for <i>exactly</i> 3 min.
After boiling	Cool in ice bath immediately.
Addition of acetic acid and iodine solutions	After addition of iodine, wait 5 min and check for complete dissolution of the copper oxide precipitate.
Addition of phosphoric acid	Carefully, with watch glass just ajar. Swirl to mix <i>slowly</i> . Keep watch glass on. Rinse underside of watch glass into flask with distilled water before titrating.
Titration	Calibrate buret at 10 mL intervals. Titrate slowly.
Calculation	Recheck.

3. Readiness To Perform

- a. Familiarization.
 - i. Phase I: Standards—NA
 - ii. Phase II: Fortified samples
 - iii. Phase III: Check samples for analyst accreditation.
- b. Acceptability criteria.
See section J.1 above.

4. Intralaboratory Check Samples

- a. System, minimum contents.
 - i. Frequency:
 - ii. Blind samples or random replicates chosen by supervisor after initial analysis.
 - iii. Records are to be maintained by analyst and reviewed by the supervisor and Laboratory QA Officer.
- b. Acceptability criteria.
If unacceptable values are obtained, then:
 - i. Stop all official analyses for that analyst.
 - ii. Investigate and identify probable cause.
 - iii. Take corrective action.
 - iv. Repeat Phase III of section J.3 if cause was analyst-related.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

5. Sample
Acceptability
and Stability

- a. Matrix: Processed meat products.
 - b. Sample receipt size, minimum: 1 lb.
 - c. Condition upon receipt: Cold, sealed from atmosphere.
 - d. Sample storage:
 - i. Time: 1 week.
 - ii. Condition: Frozen.
-

6. Sensitivity

- a. Lowest detectable level (LDL): Not determined.
 - b. Lowest reliable quantitation (LRQ): 0.3%.
 - c. Minimum proficiency level (MPL): 0.3%.
-

DEXTROSE SUCROSE MALTOSE LACTOSE

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DETERMINATIVE METHOD

A. INTRODUCTION

Theory

10 g of ground meat samples (sausages) are extracted with 55% ethanol. After filtration, the extracts are purified by passing through a C₁₈ Sep-Pak and two ion exchange resin Econo-Columns in series. The extracts after concentration are analyzed by LC using a normal phase amino column and a differential refractometer detector.

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

-
- a. Magnetic stirrer: Variable speed, Nuova 7 (Thermolyne Sybron Corp.), or equivalent.
 - b. Stirring bar: Plastic coated, 1" long.
 - c. Sugar flask with stopper: 100 mL (Kimble 28100), or equivalent.
 - d. Funnel, powder.
 - e. Pipettes: 1, 2, 3, 4, and 5 mL delivery volume.
 - f. Culture tube with stopper: 50 mL (VWR Scientific #60827-599), or equivalent.
 - g. Filter paper: Pre-pleated filter paper 18.5 cm, grade 560 (Schleicher and Schuell, Inc., Keene, NH 03431).
 - h. Volumetric flask: Low actinic, 100 mL and 500 mL.
 - i. Graduated cylinders: 50, 100, 1000 mL.
 - j. Filtering flasks: 1 and 2 liter.
 - k. Solvent clarification kit with Durapore filters: 0.45 μ m, 27 mm, but without pump and filtering flasks (Waters Associates, Milford, MA 01757).
 - l. Rotary evaporator: Buchi Model E (Brinkmann Instruments, Cantiague Road, Westberry, New York 11590), or equivalent.
 - m. C₁₈ Sep-Pak cartridge: Waters Associates.
 - n. Polypropylene Econo-Columns. 0.8 x 4 cm polypropylene column, holds 2 mL of chromatographic material and includes an internal 10 mL reservoir (Bio-Rad #731-1550).
 - o. Sample filtration apparatus: Acro LC13 disposable filter assembly with 0.45 micron Fluoropolymer membrane (Gelman Sciences Inc., 600 Wagner Road, Ann Arbor, MI 48106), or equivalent.
 - p. Limited volume inserts: Polypropylene inserts (250 μ L) (Sun Brokers Inc., P.O. Box 2230, Wilmington, NC 28402), or equivalent.
-

2. Instrumentation

-
- a. Liquid chromatograph: Waters Model No. 244, or equivalent, with Model R-401 Differential Refractometer Detector, Wisp 710B Sample Processor and Model 730 Data Module, or equivalent, or a Strip Chart Recorder, OmniScribe Chart Recorder, Model 35217-1 (Houston Instrument).
 - b. Column: 250 mm x 4 mm, Bio-Sil Amino 5S (Bio-Rad Laboratories, P.O. Box 708, 220 Maple Avenue, Rockville Centre, New York 11571).
-

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and
Solution List

-
- a. Absolute ethanol (200 proof): U.S. Industrial Chemicals Inc. (Division of National Distillers Products Corp., New Orleans, LA).
 - b. Acetonitrile: Distilled-in-glass, Non-spectra grade (Burdick and Jackson Laboratories, Inc., Muskegon, MI 49442), or equivalent.
 - c. Water: Milli-Q Water Purification System with low organic content and specific resistance ≥ 10 megohms cm^{-1} to avoid problems with extraneous interferences. (Millipore Corp.)
 - d. Petroleum ether: B.P. 30-60° C (Burdick and Jackson Laboratories, Inc., Muskegon, MI 49442), or equivalent.
 - e. Ion exchange resins:
 - i. Analytical grade cation exchange resin, AG 50W-X8, 50-100 mesh, hydrogen form (Bio-Rad Laboratories), and
 - ii. Analytical grade anion exchange resin AG 3-X4A, 20-50 mesh, chloride form (Bio-Rad Laboratories) converted to hydroxyl form by washing with ca. two volumes of 0.5N NaOH in a column at a flow rate of 1.0 mL/min and with HPLC water until chloride-free (Test for Cl^- in effluent: Acidify sample with a few drops of concentrated HNO_3 . Add a few drops of 1% AgNO_3 solution. White precipitate indicates Cl^-). (To prevent bacterial growth, resins should be stored in a refrigerator or in a cold room.)
 - f. HPLC mobile phase: Acetonitrile:water (78:22). Into a 1 L graduated cylinder add 220 mL HPLC water and dilute to volume with acetonitrile. Filter the resultant aqueous acetonitrile solvent through a Durapore filter into a 2 L filtering flask. Degas the solvent under vacuum.
-

DETERMINATIVE METHOD

D. STANDARDS

Preparation of Standards

Mixed standard solutions:

- a. Standard A: 11 mg each of dextrose, sucrose, maltose and lactose per mL. Weigh 5.5 g of each sugar (dextrose, sucrose, maltose, and lactose) into a 500 mL volumetric flask. Add ca 400 mL HPLC water, shake vigorously, and hold under running hot water to dissolve. Dilute to volume with HPLC water.
 - b. Standard B: 8.8 mg each of dextrose, sucrose, maltose, and lactose per mL. Dilute 80 mL of mixed standard A to 100 mL with HPLC water.
 - c. Standard C: 6.6 mg each of dextrose, sucrose, maltose, and lactose per mL. Dilute 60 mL of mixed standard A to 100 mL with HPLC water.
 - d. Standard D: 4.4 mg each of dextrose, sucrose, maltose, and lactose per mL. Dilute 40 mL of mixed standard A to 100 mL with HPLC water.
-

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE

1. Column Preparation

Ion exchange columns: Soak resins (refer to Section C, item e) separately in distilled water for an hour. Rinse with distilled water several times by decantation until supernatants are colorless and pH of supernatants remain unchanged. Pour cation exchange and anion exchange resins as aqueous slurries onto two separate Econo-Columns (refer to Section B, item n) until bed heights reach up to 38 mm and 14 mm respectively. Wash the columns twice with ca. 10 mL HPLC water each time. Position the cation exchange column above the anion column to allow elution in sequence. Do not let columns dry out. The columns are now ready for use.

NOTE: Purification step utilizing ion exchange resin Econo-Columns is critical. Various chemical companies supply ion exchange resins. Often these resins are considered interchangeable or equivalent. Experience with resin shows that the activity of the resin differs from batch to batch even using product from the same company. Prior to application of recovery sample onto ion exchange resin columns, several standards should be eluted to attain approximately 90-95% recovery. Bio-Rad's anion resin AG 3-X4A comes in chloride form, which must be converted to hydroxyl form to avoid formation of "inverted sugars" in the extract.

2. Sample Extraction

- a. Weigh 10.0 g of thoroughly comminuted meat product into a 100 mL sugar flask. Select a "blank" meat sample as a control and for spiking. Fortify with 0.0, 2.0, 3.0, 4.0, and 5.0 mL of mixed standard A. Each 10 g fortified sample represents 0.0, 0.22, 0.33, 0.44, and 0.55 percent dextrose, sucrose, maltose, and lactose.
- b. Add 55 mL ethanol to each sample in the flask, and add distilled water for a final liquid volume of 100 mL. (Moisture content of the meat product should be used in determining the liquid volume).
- c. Break up the lumps of meat with a clean spatula, add stirring bar, stopper, and stir on a magnetic stirrer for 10 min at medium speed.
- d. Filter about 45 mL extract into a 50 mL stoppered test tube using pre-pleated filter paper. (Stopping point. Refrigerate for overnight stopping.)
- e. Pipet a 20 mL aliquot into another 50 mL stoppered test tube.
- f. Add 15 mL pet ether and shake vigorously for a minute. Allow phases to separate by letting stand 5 min.
- g. Siphon the pet ether layer and discard.
- h. Repeat steps f-g two more times.
- i. Pass the extract through a C₁₈ Sep-Pak cartridge and collect the effluent in a 250 mL round-bottom flask (RBF).
- j. Evaporate the extract on a rotary evaporator using water bath temperature of 40° C.

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE (Continued)

-
- k. Dissolve the residue in ca. 2 mL water (refer to section C, item c), transfer onto the ion exchange column, and collect effluent in a 250 mL RBF. Rinse the flask four times with ca. 4 mL HPLC water each time and apply onto the column. Collect all the effluents in the RBF. Add 5 mL ethanol to speed up evaporation.
 - l. Evaporate the combined effluents to dryness on a rotary evaporator using a water bath temperature of 40° C. (Stopping point. Refrigerate for overnight stopping.)
 - m. Add 1.0 mL HPLC water and stopper.
 - n. Swirl on a vortex for 30 sec.
 - o. Let stand for 1 min.
 - p. Filter an aliquot through a 0.45 μ m Fluropore filter into a limited volume insert and stopper for HPLC analysis. (Stopping point. Store in refrigerator for overnight stopping.)

NOTES: The analytical range of 0.22-0.55% for all four sugars was chosen for two reasons: (1) to obtain comparable peak heights of all four sugars, and (2) to allow for the low level of maltose in the samples. The following comments are added to explain the selection of the amounts of each sugar used in preparation of the standards:

- Typically, the CSS added to the meat product contains $\geq 15\%$ maltose. Based on maximum allowable limit of 2% CSS in the product, the meat should contain $2 \times 0.15 = 0.3\%$ maltose, assuming the CSS contains 15% maltose. Therefore, 0.22, 0.33, 0.44, and 0.55% recoveries established for this method correspond to 11, 16.5, 22, and 27.5% maltose levels in CSS.
- Similarly, 0.22, 0.33, 0.44, and 0.55% lactose recoveries are equivalent to 0.44, 0.66, 0.88, and 1.10% nonfat dry milk (NFDM) based on 50% lactose in the NFDM. Since the maximum allowable limit of NFDM is 3.5%, an appropriate dilution of the sample extract is needed prior to LC analysis.
- Since dextrose and sucrose are not regulated in the meat product, if analyses of these products are requested, sample extracts should be diluted accordingly, prior to LC analysis.
- Normally, 1.0% dextrose is added to meat products. If dextrose level exceeds 1.0% in a sample, the *extract* should be analyzed for sorbitol using method DXT2, because dextrose and sorbitol coelute on the carbohydrate (amino) column.

Since NFDM, CSS/CS, dextrose, and sucrose are added substances, they are not always distributed homogeneously in the product. Therefore, a sample size of 10 g used in this method is considered more representative of the finished product.

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE (Continued)

3. Determination

Using isocratic conditions, equilibrate the entire system with mobile phase until a steady baseline is obtained at a flow rate of 1.0 mL/min and chart speed 1.5 mm/min. Using the Wisp 710B Sample Processor, inject (in duplicate) 25 μ L portions of sample extracts and mixed standards. Set range at 16X on the differential refractometer detector. Calculate concentration of each sugar in the sample as follows: using peak height and concentration of standards, construct a linear standard curve for each compound based on the formula, $y = mx + b$, where y is the peak height, x is concentration (%), m is slope, and b is y -intercept. The correlation coefficient should be ≥ 0.9900 . Calculate recovery of the spiked sample (included with every set). Recoveries should be $> 99\%$ with standard deviations ≤ 0.02 at the 0.5% level and ≤ 0.04 at the 2.0% level. The resolutions of peaks for maltose and lactose could be improved further by using two analytical columns in series. Since maltose in corn syrup solids/corn syrup (CSS/CS) varies from source to source, the same CSS/CS that has been added to the product should be analyzed simultaneously to determine the percentage of CSS/CS in the product.

4. Identification

Using the conditions of this method, the retention times (minutes) of all four sugars are as follows: dextrose, 14.25; sucrose, 20.25, maltose, 24.37; and lactose, 28.1. Identify peaks as described in Figure 1.

5. Reference

Ali, M. Sher: Simultaneous Determination of Dextrose, Sucrose, Maltose, and Lactose in Sausage Products by Liquid Chromatography, JAOAC, 71, 1097-1100, 1988.

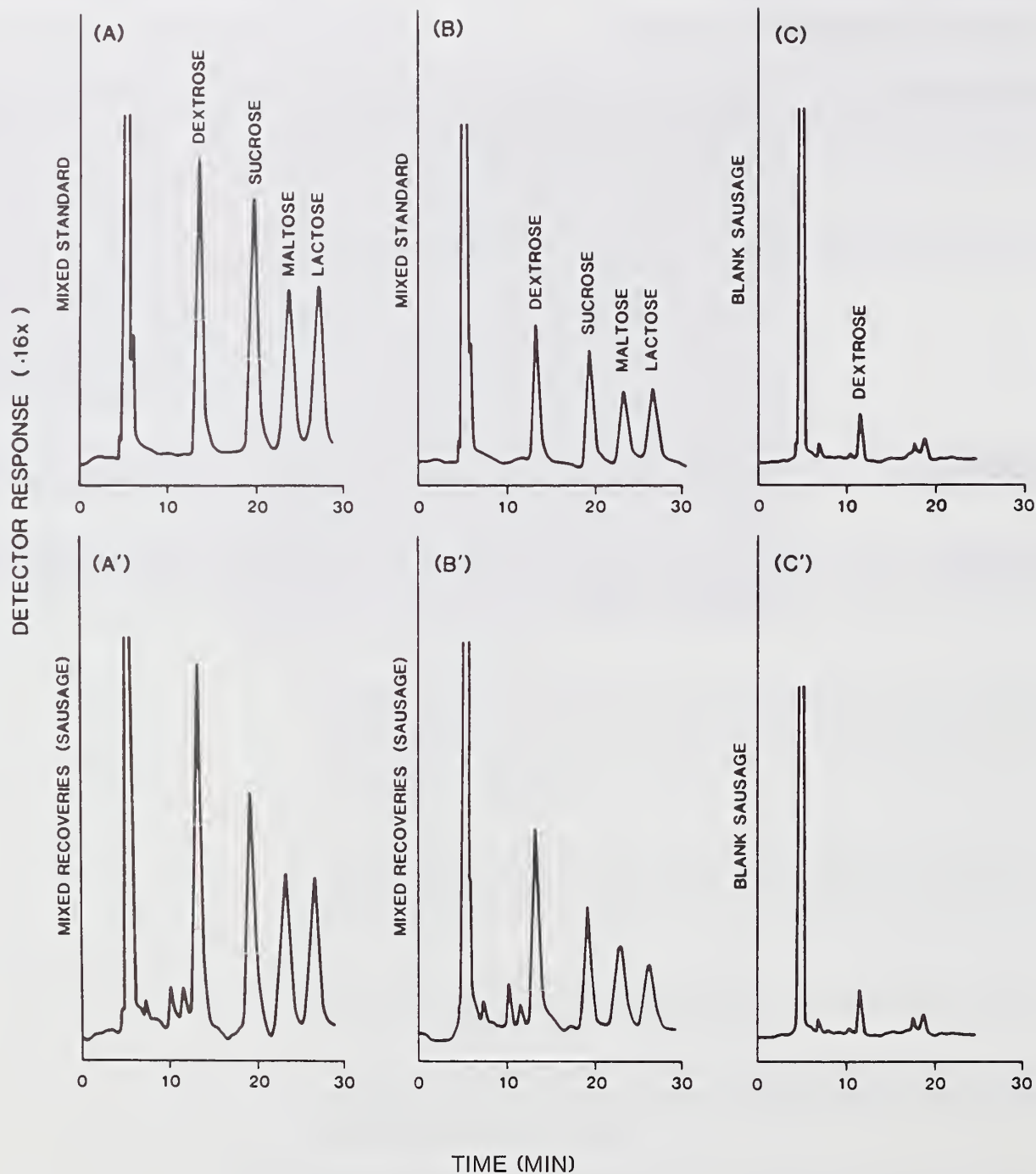


Figure 1. LC chromatograms of (A) and (B) of mixed standards of dextrose, sucrose, maltose, and lactose at the highest and lowest levels used in the method and (C) unfortified meat; (A') and (B') mixed recoveries of dextrose, sucrose, maltose, and lactose at the highest and lowest of fortifications used in the method and (C') unfortified meat. RI detector, sensitivity 16X.

DEXTROSE, MANNITOL, SORBITOL

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DETERMINATIVE METHOD

A. INTRODUCTION

Theory

Five grams of ground meat (sausage or hamburger) are extracted with 55% ethanol. After filtration, the extracts are purified by passing through C₁₈ Sep-Pak and two ion exchange columns in series. The extracts are concentrated and analyzed by LC using a cation exchange resin column and a differential refractometer detector.

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

-
- a. Magnetic stirrer, variable speed: Nuova 7 (Thermolyne Sybron Corp.) or equivalent.
 - b. Magnetic bar, plastic-coated: 1" long.
 - c. Sugar flask with stopper: 100 mL, Kimble 28100 or equivalent.
 - d. Funnel, powder.
 - e. Pipettes: 2 and 10 mL delivery volume.
 - f. Culture tube with stopper: 50 mL, VWR 60827-599 or equivalent.
 - g. Filter paper, pre-pleated: 18.5 cm, grade 560 (Schleicher and Schuell, Inc., Keene, NH 03431).
 - h. Volumetric flasks, low actinic: 100 and 500 mL.
 - i. Graduated cylinders: 50, 100, and 1000 mL.
 - j. Filtering flasks: 1 and 2 L.
 - k. Solvent clarification kit with Durapore filters: 0.45 μ m, 27 mm, but without pump and filtering flasks (Waters Associates).
 - l. Rotary evaporator: Buchi Model E (Brinkmann Instruments, Cantiague Road, Westberry, NY 11590) or equivalent.
 - m. C₁₈ Sep-Pak cartridge (Waters Associates).
 - n. Column heater: Model HPLC heater (Bio-Rad Laboratories, P.O. Box 708, 220 Maple Avenue, Rockville Centre, NY 11571) or equivalent.
 - o. Sample filtration apparatus: Acro LC13 disposable filter assembly with 0.45 micron fluoropolymer membrane (Gelman Sciences Inc., 600 South Wagner Road, Ann Arbor, MI 48106) or equivalent.
 - p. Limited volume inserts, polypropylene: 250 μ L, Sun Brokers, Inc., P.O. Box 2230, Wilmington, NC 28402 or equivalent.
-

2. Instrumentation

-
- a. Liquid chromatograph: Model 244, Waters Associates, with Model R-401 Differential Refractometer Detector, Wisp 710B Sample Processor and Model 730 Data Module, or equivalents, or a Strip Chart Recorder, Omniscribe Chart Recorder, Model 35217-1 (Houston Instrument) or equivalent.
 - b. Column: 300 mm \times 7.8 mm 8% crosslinked cation exchange resin column, Aminex HPX-87C (Bio-Rad Laboratories).
 - c. Polypropylene columns: 0.8 \times 4 cm Econo-Column, holds 2 mL of chromatographic medium and includes an internal 10 mL reservoir (Bio-Rad catalog #731-1550).
-

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

**Reagent and
Solution List**

- a. Absolute ethanol (200 proof), U. S. Industrial Chemicals, Inc. (Division of National Distillers Products Corp., New Orleans, LA).
- b. Water: Milli-Q Water Purification System with low organic content and a specific resistance of 10 megohms cm^{-1} or greater to avoid problems with extraneous interferences (Millipore Corp.).
- c. Petroleum ether: B.P. 30°-60° C, Burdick and Jackson Laboratories, Inc., Muskegon, MI 49442 or equivalent.
- d. Analytical grade cation exchange resin, AG 50W-X8, 50-100 mesh, hydrogen form (Bio-Rad Laboratories).
- e. Analytical grade anion exchange resin, AG 3-X4A, 20-50 mesh, chloride form (Bio-Rad Laboratories) converted to hydroxyl form by washing with ca. 2 volumes of 0.5N NaOH in a column at a flow rate of 1.0 mL/min. The column is washed with HPLC water until the effluent is chloride-free. (Test for Cl^- in effluent: Acidify sample with a few drops of conc. HNO_3 . Add a few drops of 1% AgNO_3 solution. White precipitate indicates Cl^- .)

NOTE: To prevent bacterial growth, resins should be stored in a refrigerator or in a cold room.

- f. HPLC mobile phase: Filter HPLC water through a Durapore filter into a 2 L filtering flask and de-gas under vacuum.
-

DETERMINATIVE METHOD

D. STANDARDS

Preparation of Standards

Mixed standard solutions.

- a. Standard A: 20 mg each of dextrose, mannitol, and sorbitol per mL. Weigh 10 g of each sugar into a 500 mL volumetric flask. Add ca. 400 mL HPLC water. Shake vigorously and hold under running hot water to dissolve. Dilute to volume with HPLC water.
 - b. Standard B: 15 mg each of dextrose, mannitol, and sorbitol per mL. Dilute 75 mL of mixed standard A to 100 mL with HPLC water.
 - c. Standard C: 10 mg each of dextrose, mannitol, and sorbitol per mL. Dilute 50 mL of mixed standard A to 100 mL with HPLC water.
 - d. Standard D: 5.0 mg each of dextrose, mannitol, and sorbitol per mL. Dilute 25 mL of mixed standard A to 100 mL with HPLC water.
-

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE

1. Column Preparation

Ion exchange columns: Soak resins (refer to Section C, items d and e) separately in distilled water for 1 hr. Rinse with distilled water several times by decantation until supernatants are colorless and pH of supernatants remain constant. Pour cation exchange and anion exchange resins as aqueous slurries into two separate Econo-Columns until bed heights reach up to 38 mm and 14 mm respectively. Wash the columns with HPLC water. Position the cation exchange column above the anion exchange column to allow elution in sequence. Do not let columns dry out.

NOTE: Purification step utilizing ion exchange resin Econo-Columns is critical. Various chemical companies supply ion exchange resins. Often these resins are considered interchangeable or equivalent. Experience with resin shows that the activity of the resin differs from batch to batch even using product from the same company. Prior to application of recovery sample onto ion exchange resin columns, several standards should be eluted to attain approximately 90-95% recovery. Bio-Rad's anion resin AG 3-X4A comes in chloride form, which must be converted to hydroxyl form to avoid formation of "inverted sugars" in the extract, if the sample contains added sucrose.

2. Sample Extraction

- a. Weigh 5.0 g of thoroughly comminuted meat product into 100 mL sugar flask. Select a "blank" meat sample as a control and for spiking. Fortify with 10 mL of each mixed standard (A, B, C, and D) per 5.0 g of samples, representing 4, 3, 2, and 1% dextrose, mannitol, and sorbitol respectively.

NOTE: The analytical range of 1.0-4.0% for all dextrose, mannitol, and sorbitol was chosen because of the limitation of 2% sorbitol in the finished product.

- b. Add 55 mL ethanol to the sample in the flask. Use distilled water for a final liquid volume of 100 mL. (Moisture content of the meat product should be used in determining the liquid volume.)
- c. Break up the lumps of meat with a clean spatula, add a magnetic bar, stopper, and stir on a magnetic stirrer for 10 min at medium speed.
- d. Filter about 45 mL extract into a 50 mL stoppered test tube using prepleated filter paper. (**Stopping point:** Refrigerate for stopping overnight.)
- e. Pipet 20 mL filtrate into another 50 mL stoppered test tube.
- f. Add 15 mL petroleum ether and shake vigorously for 1 min. Let stand for 5 min to separate the layers.
- g. Siphon the petroleum ether layer and discard.
- h. Repeat steps f and g two more times.
- i. Pass the extract through a C₁₈ Sep-Pak cartridge and collect the effluents in a 250 mL round-bottom flask.
- j. Evaporate the extract on a rotary evaporator with a water bath temperature of 40° C.

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE (Continued)

-
- k. Dissolve the residue in ca. 2 mL HPLC water. Apply to the ion exchange column and collect the effluent in a 250 mL round-bottom flask. Rinse the flask 4 times with ca. 4 mL HPLC water each time and apply to the column. Collect all the effluents in the round-bottom flask. Add ca. 5 mL ethanol to speed up evaporation.
 - l. Evaporate the combined effluents on a rotary evaporator at a water bath temperature of 40° C. (**Stopping point:** Refrigerate if stopping overnight.)
 - m. Add 2.0 mL HPLC water and stopper.
 - n. Swirl on a vortex several times.
 - o. Let stand for 1 min.
 - p. Filter an aliquot through a 0.45 μ m Fluoropore filter into a limited volume insert and stopper for HPLC analysis. (**Stopping point:** Refrigerate if stopping overnight.)

NOTE: With each set of samples to be analyzed, process one reagent blank, one control, and one blank sample fortified with mixed standard C. (This represents 2% dextrose, mannitol, and sorbitol.)

3. Determination

- a. Raise the temperature of the column to 75° C while the solvent is pumped through it.
- b. Using isocratic conditions, equilibrate the entire system with mobile phase until a steady baseline is obtained (about 30-45 min) at a flow rate of 0.6 mL/min and chart speed 1.5 mm/min.
- c. Using the Wisp 710B Sample Processor, inject (in duplicate) 25 μ L portions of sample extracts and mixed standards. Set range at 32X on Differential Refractometer Detector.
- d. Calculate concentrations of each sugar in the sample as follows:
 - i. Using peak height and concentration of standards, construct a linear standard curve for each compound based on the formula $y = mx + b$, where y is the peak height, x is concentration (%), m is slope, and b is y intercept.
 - ii. Tabulate the results on the worksheets provided. The correlation coefficient should be $\geq .9900$. Calculate recovery of the spiked sample (included with every set). Recoveries should be $> 99\%$ with standard deviations ≤ 0.02 at the 0.5% level and ≤ 0.04 at the 2.0% level.

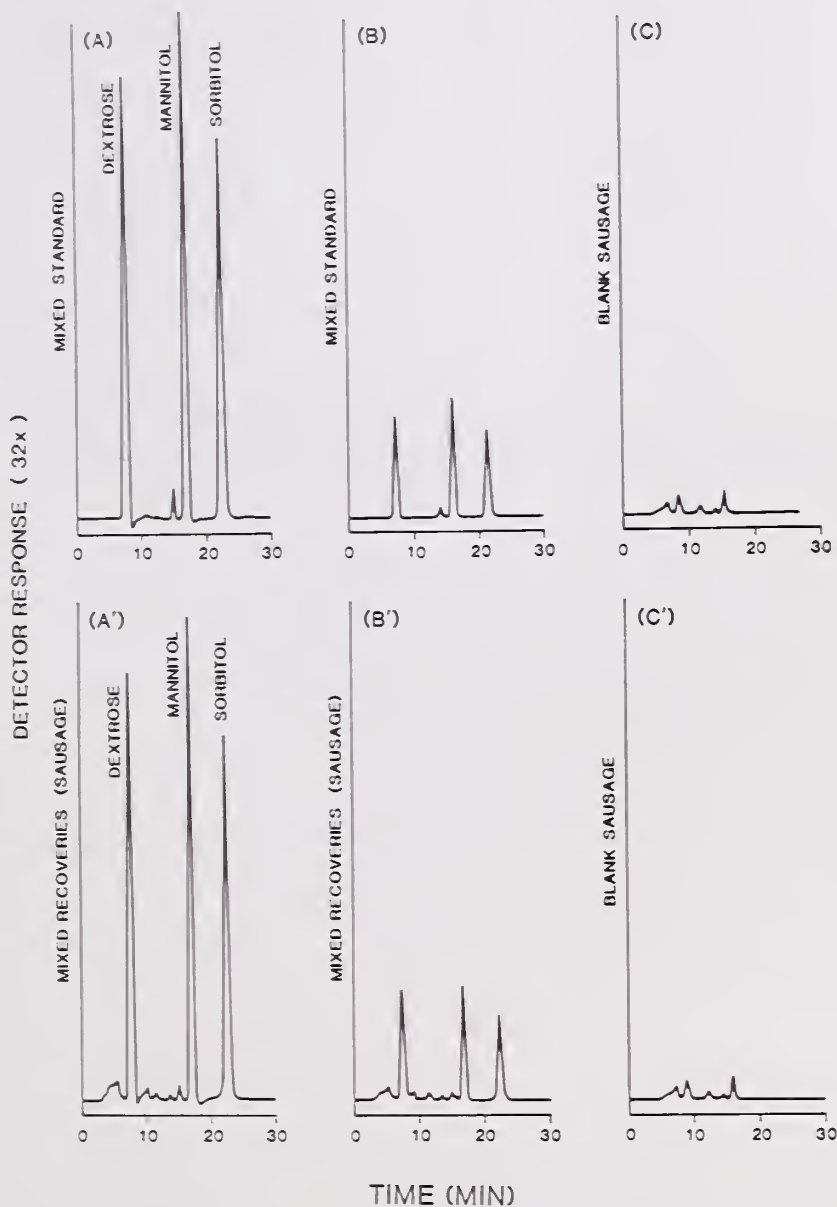
4. Identification

Using the conditions of this method, the retention times (min) of all sugars are as follows: dextrose, 8.50; mannitol, 17.00; and sorbitol, 22.52. Identify peaks as described in Figure 1 on the facing page.

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE (Continued)

5. Figure 1



LC chromatograms of (A) and (B) mixed standards of dextrose, mannitol, and sorbitol at the highest and lowest levels used in the method and (C) unfortified meat: (A') and (B') mixed recoveries of dextrose, mannitol, and sorbitol at the highest and lowest of fortifications used in the method and (C') unfortified meat. RI detector, sensitivity 16X.

FAT (ETHER EXTRACTION)

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DETERMINATIVE METHOD

A. INTRODUCTION

Theory

This method involves a partial drying of a weighed sample prior to a Soxhlet extraction. The extracted fat is weighed and the fat content calculated.

Sample weights are obtained by difference in order to minimize changes in the moisture content, which will adversely affect the fat content. Regardless of which procedure is used, it is important that sand be incorporated with the sample before drying. The purpose of the sand is to create a greater surface area, necessary to remove moisture and prevent entrapment of fat.

Excessive drying may oxidize the fat and give high results.

DETERMINATIVE METHOD

B. EQUIPMENT

Apparatus

-
- a. Thimbles: fat extraction 33 × 80 mm, Whatman #2800-338 or equivalent.
 - b. Soxhlet extraction apparatus: id of extraction tube, 40 mm, Fisher #09-551B or equivalent.
 - c. Heating mantles, VWR #33749-324 (6-unit manifold) or equivalent.
 - d. Filter paper: 9 cm Whatman #541 or equivalent.
 - e. Aluminum dishes, disposable: approximately 60 mm diameter × 18 mm depth, Fisher #08-732 or equivalent.
 - f. Glass beads, hollow, perforated: 4 mm diameter.
 - g. Oven, mechanical convection.
-

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

- | |
|---|
| a. Petroleum ether: AOAC 945.16, 15th Edition. |
| b. Sand, washed and ignited: CAS 74808-60-7, VWR #JT 3382-1, or equivalent. |

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE

Determination

- a. (1) Accurately weigh, by difference, 3 to 4 g of sample into a thimble lined with a circle of filter paper and containing a small amount of sand.

Mix sand and sample with a glass rod, wipe glass rod with filter paper strips, and place strips in thimble.

Place thimble and contents in 50 mL beaker and dry in a mechanical convection oven for 6 hr at 100-102° C, or for 1-1/2 hr at 125° C. Proceed as in b. below.

- (2) Accurately weigh, by difference, 3 to 4 g of sample into a small disposable aluminum dish.

Add a small amount of sand, and with the aid of a small aluminum or glass paddle, spread the mixture across the bottom of the dish.

Dry as in a. above, roll edges of dish, and insert into a thimble. Proceed as in b. below.

- b. Accurately weigh an extraction flask containing a few glass beads.
- c. Extract the sample contained in the thimble from a (1) or (2) above with petroleum ether for 4 hr at a condensation rate of at least 5-6 drops per sec, in a Soxhlet extraction apparatus. (If sample has been dried as in a (1) above, rinse the 50 mL beaker with three 10 mL portions of petroleum ether and add rinsings to the extraction tube.)
- d. Upon completion of the extraction, place flask on a steam bath and evaporate the petroleum ether until no odor of it is detectable. Swirl flask initially to avoid boil-over.
- e. Dry flask and contents in a mechanical convection oven at 100° C for time required to obtain constant weight. Cool in desiccator to room temperature and weigh.

NOTE: Laboratory must have data available to support time used to obtain constant weight. If these data are available, analysts may use the time rather than remove, cool, and weigh several times for each sample.

DETERMINATIVE METHOD

G. CALCULATIONS

1. Procedure

$$\text{Fat content, percent} = \frac{100 (B - C)}{A}$$

A = Sample weight

B = Weight of flask after extraction

C = Weight of flask prior to extraction

2 Reference

Official Methods of Analysis of the Association of Official Analytical Chemists, 960.39, 15th Edition.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Fat Determination (Ether Extraction).		
2. Required Protective Equipment	Safety glasses, lab coat, and heat-resistant gloves.		
3. Procedure Steps		<u>Hazards</u>	<u>Recommended Safe Procedures</u>
	Extract the sample with petroleum ether for 4 hr at a condensation rate—etc.	Petroleum ether—flammable liquid	Work in hood located in well-ventilated area. Keep away from sparks or open flame.
	At the completion of extraction, place flask on a <i>steam bath</i> and evaporate the petroleum ether until no characteristic odor of it is detectable.	See above.	Allow evaporation to continue several minutes beyond the point where no ether is observed in order to minimize personal exposure.
4. Disposal Procedures	Extracted meat waste and extraction thimbles.	Residual petroleum ether vapor.	Store in cool, well-ventilated area awaiting disposal.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standard

<i>Compound</i>	<i>Analytical Range (%)</i>	<i>Repeatability CV %</i>	<i>Reproducibility CV %</i>
Fat	‡	<0.63*	<0.66*

‡ Limit may vary due to sample and aliquot sizes and sample type.
* Standard deviation.

2. Critical Control Points and Specifications

<i>Record</i>	<i>Acceptable Control</i>
Sample size	3.5 g \pm 0.5 g.
Predry	In oven 6 hr at 100-102° C or for 1½ hr at 125° C \pm 1° C.
Solvent Extractables	< 0.004 g per 100 mL. Record in "Standards Book."
Sand—Extractables	< 0.004 g per 5 g. Record in "Standards Book."
Extraction time and rate	4 hr \pm 10 min, at least 5-6 drops per sec via Soxhlet (80-100 cycles/4 hr; ~ 1 cycle/3 min).
Drying time and temperature after extraction	100° C \pm 1° C for time required to obtain constant weight. (Data must be available to prove validity of time used.)
Calculation	Recheck.

3. Readiness To Perform

- a. Familiarization.
 - i. Phase I: Standards—NA.
 - ii. Phase II: Fortified samples—Random replicates of previously analyzed samples.
 - iii. Phase III: Check samples for analyst accreditation.
- b. Acceptability criteria.
See section J.1 above.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

4. Intralaboratory Check Samples

- a. System, minimum contents.
 - i. Frequency: 1 per week, not to exceed 20% of samples.
 - ii. Blind samples or random replicates chosen by supervisor after initial analysis.
 - iii. Records are to be maintained by analyst and reviewed by the supervisor and Laboratory QA Officer.
 - b. Acceptability criteria: Refer to section J.1 above.

If unacceptable values are obtained, then:

 - i. Stop all official analyses for that analyst.
 - ii. Investigate and identify probable cause.
 - iii. Take corrective action.
 - iv. Repeat Phase III of section J.3 if cause was analyst-related.
-

5. Interlaboratory Check Sample Program

- a. Laboratory responsibility: Analyze samples and report results promptly; maintain appropriate records.
 - b. Chemistry Division responsibility: Evaluate data and report promptly to participants.
 - c. Acceptability criteria: Refer to section J.1 above.
-

6. Sample Acceptability and Stability

- a. Matrix: Meat, poultry, and processed products.
 - b. Sample receipt size, minimum: Size varies with sampling program. See Inspection Manual, Part 23.
 - c. Condition upon receipt: Cold and sealed from air.
 - d. Sample storage:
 - i. Time: 1 week.
 - ii. Condition: 4° C.
-

7. Sensitivity

- a. Lowest detectable level (LDL): NA.
 - b. Lowest reliable quantitation (LRQ): 0.5%.
 - c. Minimum proficiency level (MPL): 0.5%.
-

FAT (SPECIFIC GRAVITY)

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DETERMINATIVE METHOD

A. INTRODUCTION

Theory

This method utilizes the Foss-Let fat analyzer, which is based on the principle of a density measurement of a tetrachloroethylene extract of the fat in a magnetic float cell. The sample is first homogeneously prepared in a "reactor," which breaks down the sample, squeezing out the oil while simultaneously absorbing the moisture in the sample with calcium sulfate.

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

-
- a. Top-loading balance, capable of weighing $45\text{ g} \pm 0.1\text{ g}$ samples.
 - b. 7 cm diameter filter paper, Schleicher & Schuell (S & S), No. 589, or equivalent.
-

2. Instrumentation

Foss-Let apparatus and accessories: dispenser, cooler, "reactor," and measuring unit (Foss America, Inc., P.O. Box 504, Fishkill, NY, 12524).

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

**Reagent and
Solution List**

-
- a. Tetrachloroethylene (perchloroethylene), available from local dry cleaning suppliers.
 - b. Calcium sulfate, anhydrous, or plaster of paris (available from local hardware stores).
 - c. Foss-Let mineral oil (specific gravity at 23° C, -0.915).
-

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE

1. Determination

- a. Allow Foss-Let potentiometer to obtain maximum stability by turning the instrument on with chamber filled with perchloroethylene at least two hours before operation.

CAUTION: Perchloroethylene vapors are harmful to inhale and handling of this liquid should be confined to a fume hood.

- b. Daily calibrate the Foss-Let potentiometer by using perchloroethylene to set the zero point before samples are analyzed. The zero point must read 000 ± 001 ; adjust side knob of instrument using a screw driver. Calibrate the instrument by using Foss-Let mineral oil (specific gravity at $23^{\circ}\text{C} - 0.915$) to set control for 50% fat point at 850.0 reading. The swimmer must rise at a digital setting of 850 ± 003 . Adjust side knob of instrument using a screw driver. Record zero point and 50% fat point readings in Standards Record Notebook.
- c. Daily, adjust compensator of perchloroethylene dispenser to correspond with the ambient temperature.
- d. Weigh a $45.0\text{ g} \pm 0.1\text{ g}$ sample into a stainless steel extraction-chamber cup.
- e. Set brass hammer with ring etched bottom on the spindle in the cup. Check to assure the bore of the hammer is less than or equal to 11 mm diameter or do not use.
- f. Add 80 g of plaster of paris (approximately 3 oz) or 60 g of anhydrous calcium sulfate to the cup. For very wet samples, more may be required to obtain clear filtrate (not milky).
- g. Dispense perchloroethylene $120\text{ mL} \pm 0.1\text{ mL}$ into the cup using the dispenser unit. Place the cover tightly on the cup.
- h. Put the extraction cup onto the "reactor" and shake for 2 min.
- i. Assemble the filtration device by first placing a 7 cm circle of S & S No. 589 filter paper into perforated base. Place filtration device at top of measuring chamber of Foss-Let analyzer.
- j. Remove the cup from the shaker and lift the cover. Pour the liquid extract through the assembled filtration device into the Foss-Let potentiometer measuring chamber. Filtrate must be clear, not cloudy. Allow the filtration to continue at least until the extract appears in the overflow clear tubing to the waste container and 10 mL of filtrate is retained in the measuring chamber.
- k. Remove the filtration device, slide the viewing lens to the right into position, and allow the temperature control lamp to switch off before taking a reading of the specific gravity of the crude fat extract in perchloroethylene. If the temperature in the measuring chamber is too low, the temperature control lamp is on. If the lamp is flashing, the temperature is too high.

WARNING: Do not move the viewing lens to the left since this automatically releases the drain valve.

DETERMINATIVE METHOD

F. ANALYTIC PROCEDURES (Continued)

-
- l. When the measuring chamber is at the correct temperature, push the black swimmer reset button and view the movements of the hydrometer through the viewing lens. Turn the digital knob clockwise (towards a higher reading) slowly, just until swimmer rises. Repeat reading 3 times and record the mean digital readout on a worksheet or in a workbook.
 - m. Convert the digital readout figure to a percent fat reading (2 decimal places) using the conversion table (e.g., a readout of 99 is equivalent to 5.00% fat.) Round the percent fat reading to the nearest 0.1%.
 - n. Move the viewing lens to the left and allow the retained filtrate to drain into a plastic 10 L waste bottle. Rinse the measuring chamber three times with approximately 40 mL of clean perchloroethylene. Depress the red drain valve button so the waste will drain completely into the 10 L container.
 - o. Clean the extraction cup, cup cover, brass hammer, and filtration unit thoroughly, assuring proper waste disposal procedures under the fume hood. Allow the washed items to thoroughly dry before reuse.
-

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE (Continued)

2. Foss-Let Units

Conversion Table										
	0	1	2	3	4	5	6	7	8	9
	% Fat Content									
0	0	0.05	0.10	0.15	0.20	0.25	0.30	0.35	0.40	0.45
10	0.50	0.55	0.60	0.65	0.70	0.75	0.80	0.85	0.90	0.95
20	1.00	1.05	1.10	1.15	1.20	1.25	1.30	1.35	1.40	1.45
30	1.50	1.55	1.60	1.65	1.70	1.75	1.80	1.85	1.90	1.95
40	2.00	2.05	2.10	2.15	2.20	2.25	2.30	2.35	2.40	2.45
50	2.50	2.55	2.60	2.65	2.70	2.75	2.80	2.85	2.90	2.95
60	3.00	3.05	3.10	3.15	3.20	3.25	3.30	3.35	3.40	3.45
70	3.50	3.55	3.60	3.65	3.70	3.75	3.80	3.85	3.90	3.95
80	4.00	4.05	4.10	4.15	4.20	4.25	4.30	4.35	4.40	4.45
90	4.55	4.60	4.65	4.70	4.75	4.80	4.85	4.90	4.95	5.00
100	5.05	5.10	5.15	5.20	5.25	5.30	5.35	5.40	5.45	5.50
110	5.55	5.60	5.65	5.70	5.75	5.80	5.85	5.90	5.95	6.00
120	6.10	6.15	6.20	6.25	6.30	6.35	6.40	6.45	6.50	6.55
130	6.60	6.65	6.70	6.75	6.80	6.85	6.90	6.95	7.00	7.05
140	7.10	7.15	7.20	7.25	7.30	7.40	7.45	7.50	7.55	7.60
150	7.65	7.70	7.75	7.80	7.85	7.90	7.95	8.00	8.05	8.10
160	8.15	8.20	8.25	8.30	8.35	8.40	8.50	8.55	8.60	8.65
170	8.70	8.75	8.80	8.85	8.90	8.95	9.00	9.05	9.10	9.15
180	9.20	9.25	9.30	9.35	9.40	9.50	9.55	9.60	9.65	9.70
190	9.75	9.80	9.85	9.90	9.95	10.00	10.05	10.10	10.15	10.25
200	10.30	10.35	10.40	10.45	10.50	10.55	10.60	10.65	10.70	10.75
210	10.80	10.85	10.90	10.95	11.05	11.10	11.15	11.20	11.25	11.30
220	11.35	11.40	11.45	11.50	11.55	11.60	11.65	11.75	11.80	11.85
230	11.90	11.95	12.00	12.05	12.10	12.15	12.20	12.25	12.30	12.40
240	12.45	12.50	12.55	12.60	12.65	12.70	12.75	12.80	12.85	12.90

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE (Continued)

	Conversion Table									
	0	1	2	3	4	5	6	7	8	9
	% Fat Content									
250	12.95	13.05	13.10	13.15	13.20	13.25	13.30	13.35	13.40	13.45
260	13.50	13.55	13.65	13.70	13.75	13.80	13.85	13.90	13.95	14.00
270	14.05	14.10	14.15	14.25	14.30	14.35	14.40	14.45	14.50	14.55
280	14.60	14.65	14.70	14.80	14.85	14.90	14.95	15.00	15.05	15.10
290	15.15	15.20	15.30	15.35	15.40	15.45	15.50	15.55	15.60	15.65
300	15.70	15.75	15.85	15.90	15.95	16.00	16.05	16.10	16.15	16.20
310	16.25	16.35	16.40	16.45	16.50	16.55	16.60	16.65	16.70	16.80
320	16.85	16.90	16.95	17.00	17.05	17.10	17.15	17.20	17.30	17.35
330	17.40	17.45	17.50	17.55	17.60	17.65	17.75	17.80	17.85	17.90
340	17.95	18.00	18.05	18.10	18.20	18.25	18.30	18.35	18.40	18.45
350	18.50	18.60	18.65	18.70	18.75	18.80	18.85	18.90	18.95	19.05
360	19.10	19.15	19.20	19.25	19.30	19.35	19.45	19.50	19.55	19.60
370	19.65	19.70	19.75	19.85	19.90	19.95	20.00	20.05	20.10	20.15
380	20.25	20.30	20.35	20.40	20.45	20.50	20.55	20.65	20.70	20.75
390	20.80	20.85	20.90	20.95	21.05	21.10	21.15	21.20	21.25	21.30
400	21.40	21.45	21.50	21.55	21.60	21.65	21.70	21.80	21.85	21.90
410	21.95	22.00	22.05	22.15	22.20	22.25	22.30	22.35	22.40	22.50
420	22.55	22.60	22.65	22.70	22.75	22.85	22.90	22.95	23.00	23.05
430	23.10	23.20	23.25	23.30	23.35	23.40	23.45	23.55	23.60	23.65
440	23.70	23.75	23.80	23.90	23.95	24.00	24.05	24.10	24.15	24.25
450	24.30	24.35	24.40	24.45	24.55	24.60	24.65	24.70	24.75	24.80
460	24.90	24.95	25.00	25.05	25.10	25.20	25.25	25.30	25.35	25.40
470	25.50	25.55	25.60	25.65	25.70	25.75	25.85	25.90	25.95	26.00
480	26.05	26.15	26.20	26.25	26.30	26.35	26.45	26.50	26.55	26.60
490	26.65	26.75	26.80	26.85	26.90	26.95	27.00	27.10	27.15	27.20

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE (Continued)

	Conversion Table									
	0	1	2	3	4	5	6	7	8	9
	% Fat Content									
500	27.25	27.35	27.40	27.45	27.50	27.55	27.65	27.70	27.75	27.80
510	27.85	27.95	28.00	28.05	28.10	28.15	28.25	28.30	28.35	28.40
520	28.50	28.55	28.60	28.65	28.70	28.80	28.85	28.90	28.95	29.00
530	29.10	29.15	29.20	29.25	29.35	29.40	29.45	29.50	29.55	29.65
540	29.70	29.75	29.80	29.90	29.95	30.00	30.05	30.10	30.20	30.25
550	30.30	30.35	30.45	30.50	30.55	30.60	30.70	30.75	30.80	30.85
560	30.90	31.00	31.05	31.10	31.15	31.25	31.30	31.35	31.40	31.50
570	31.55	31.60	31.65	31.75	31.80	31.85	31.90	32.00	32.05	32.10
580	32.15	32.20	32.30	32.35	32.40	32.45	32.55	32.60	32.65	32.70
590	32.80	32.85	32.90	32.95	33.05	33.10	33.15	33.20	33.30	33.35
600	33.40	33.50	33.55	33.60	33.65	33.75	33.80	33.85	33.90	34.00
610	34.05	34.10	34.15	34.25	34.30	34.35	34.40	34.50	34.55	34.60
620	34.65	34.75	34.80	34.85	34.95	35.00	35.05	35.10	35.20	35.25
630	35.30	35.35	35.45	35.50	35.55	35.65	35.70	35.75	35.80	35.90
640	35.95	36.00	36.05	36.15	36.20	36.25	36.35	36.40	36.45	36.50
650	36.60	36.65	36.70	36.80	36.85	36.90	36.95	37.05	37.10	37.15
660	37.25	37.30	37.35	37.40	37.50	37.55	37.60	37.70	37.75	37.80
670	37.85	37.95	38.00	38.05	38.15	38.20	38.25	38.35	38.40	38.45
680	38.50	38.60	38.65	38.70	38.80	38.85	38.90	39.00	39.05	39.10
690	39.15	39.25	39.30	39.35	39.45	39.50	39.55	39.65	39.70	39.75
700	39.85	39.90	39.95	40.00	40.10	40.15	40.20	40.30	40.35	40.45
710	40.50	40.55	40.60	40.70	40.75	40.80	40.90	40.95	41.00	41.10
720	41.15	41.20	41.30	41.35	41.40	41.45	41.55	41.60	41.65	41.75
730	41.80	41.85	41.95	42.00	42.05	42.15	42.20	42.25	42.35	42.40
740	42.45	42.55	42.60	42.65	42.75	42.80	42.85	42.95	43.00	43.05

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE (Continued)

	Conversion Table									
	0	1	2	3	4	5	6	7	8	9
	% Fat Content									
750	43.15	43.20	43.30	43.35	43.40	43.50	43.55	43.60	43.70	43.75
760	43.80	43.90	43.95	44.00	44.10	44.15	44.20	44.30	44.35	44.40
770	44.50	44.55	44.60	44.70	44.75	44.80	44.90	44.95	45.05	45.10
780	45.15	45.25	45.30	45.35	45.45	45.50	45.55	45.65	45.70	45.80
790	45.85	45.90	46.00	46.05	46.10	46.20	46.25	46.35	46.40	46.45
800	46.55	46.60	46.65	46.75	46.80	46.85	46.95	47.00	47.10	47.15
810	47.20	47.30	47.35	47.45	47.50	47.55	47.65	47.70	47.75	47.85
820	47.90	48.00	48.05	48.10	48.20	48.25	48.35	48.40	48.45	48.55
830	48.60	48.65	48.75	48.80	48.90	48.95	49.05	49.10	49.15	49.25
840	49.30	49.35	49.45	49.50	49.60	49.65	49.70	49.80	49.85	49.95
850	50.00	50.05	50.15	50.20	50.30	50.35	50.40	50.50	50.55	50.65
860	50.70	50.75	50.85	50.90	51.00	51.05	51.15	51.20	51.25	51.35
870	51.40	51.50	51.55	51.60	51.70	51.75	51.85	51.90	52.00	52.05
880	52.10	52.20	52.25	52.35	52.40	52.50	52.55	52.60	52.70	52.75
890	52.85	52.90	53.00	53.05	53.10	53.20	53.25	53.35	53.40	53.50
900	53.55	53.60	53.70	53.75	53.85	53.90	54.00	54.05	54.15	54.20
910	54.25	54.35	54.40	54.50	54.55	54.65	54.70	54.80	54.85	54.90
920	55.00	55.05	55.15	55.20	55.30	55.35	55.45	55.50	55.55	55.65
930	55.70	55.80	55.85	55.95	56.00	56.10	56.15	56.25	56.30	56.40
940	56.45	56.50	56.60	56.65	56.75	56.80	56.90	56.95	57.05	57.10
950	57.20	57.25	57.35	57.40	57.50	57.55	57.65	57.70	57.75	57.85
960	57.90	58.00	58.05	58.15	58.20	58.30	58.35	58.45	58.50	58.60
970	58.65	58.75	58.80	58.90	58.95	59.05	59.10	59.20	59.25	59.35
980	59.40	59.50	59.55	59.65	59.70	59.80	59.85	59.95	60.00	60.10

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Fat Determination (Specific Gravity).											
2. Required Protective Equipment	Safety glasses, plastic gloves, and lab coat.											
3. Procedure Steps	<table><tr><th></th><th><u>Hazards</u></th><th><u>Recommended Safe Procedures</u></th></tr><tr><td>C. Reagents</td><td></td><td></td></tr><tr><td>Dispense perchloroethylene 120 mL ± 0.1 mL into cup using dispenser unit.</td><td>Perchloroethylene vapors are toxic and corrosive (suspected carcinogen).</td><td>After sample weighing, all other operations should be performed in a fume hood.</td></tr></table>				<u>Hazards</u>	<u>Recommended Safe Procedures</u>	C. Reagents			Dispense perchloroethylene 120 mL ± 0.1 mL into cup using dispenser unit.	Perchloroethylene vapors are toxic and corrosive (suspected carcinogen).	After sample weighing, all other operations should be performed in a fume hood.
	<u>Hazards</u>	<u>Recommended Safe Procedures</u>										
C. Reagents												
Dispense perchloroethylene 120 mL ± 0.1 mL into cup using dispenser unit.	Perchloroethylene vapors are toxic and corrosive (suspected carcinogen).	After sample weighing, all other operations should be performed in a fume hood.										
4. Disposal Procedures	Extracted meat waste, CaSO ₄	Residual perchloroethylene vapor	Store in cool, well-ventilated area awaiting disposal.									
	Perchloroethylene waste	Perchloroethylene	Store in a prescribed solvent container that is prominently labelled and kept in cool, well-ventilated area awaiting disposal.									

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standard	Compound	Analytical Range (%)	Repeatability CV %	Reproducibility CV %
	Fat	‡	<0.63*	<0.66*
‡ Limit may vary due to sample and aliquot sizes and sample type. * Standard deviation.				
2. Critical Control Points and Specifications	Record		Acceptable Control	
	Instrument warmup		2 hours, just prior to use.	
	Instrument calibration		Zero point = 000 ± 001 50% standard reading = 850.0 ± 003	
	Dispenser compensator adjustment		Ambient room temperature	
	Sample weight		45.0 g ± 0.1 g.	
	Hammer bore		≤11 mm.	
	Anhydrous calcium sulfate or plaster of paris		Sufficient amount to obtain clear (not milky) filtrate.	
	Perchloroethylene		120 mL ± 0.1 mL.	
	Extraction		Not <2 min; no cloudy extract.	
	Instrument readings		Temperature control lamp off and 3 readings within ± 0.2.	
3. Readiness To Perform	a. Familiarization.			
	i. Phase I: Standards—NA.			
	ii. Phase II: Use ether-extracted samples to determine acceptable work.			
	iii. Phase III: Check samples for analyst accreditation.			
	b. Acceptability criteria.			
	See section J.1 above.			

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

4. Intralaboratory Check Samples

- a. System, minimum contents.
 - i. Frequency: 1 per day.
 - ii. Blind samples or random replicates chosen by supervisor after initial analysis.
 - iii. Records are to be maintained by analyst and reviewed by the supervisor and Laboratory QA Officer.
 - b. Acceptability criteria.

If unacceptable values are obtained, then:

 - i. Stop all official analyses for that analyst.
 - ii. Investigate and identify probable cause.
 - iii. Take corrective action.
 - iv. Repeat Phase III of section J.3 if cause was analyst-related.
-

5. Sample Acceptability and Stability

- a. Matrix: Ground beef, processed meat products.
 - b. Sample receipt size, minimum: 12 oz.
 - c. Condition upon receipt: Cold and sealed from air.
 - d. Sample storage:
 - i. Time: 1 month.
 - ii. Condition: 4° C.
-

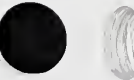
6. Sensitivity

- a. Lowest detectable level (LDL): NA.
 - b. Lowest reliable quantitation (LRQ): 0.05%.
 - c. Minimum proficiency level (MPL): 0.5%.
-

FORMALDEHYDE

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DETERMINATIVE METHOD

A. INTRODUCTION

Theory

A violet color produced by the interaction of formaldehyde and 4, 5-dihydroxy-2, 7-naphthalene-disulfonic acid follows Beer's Law. The interfering substances (such as carbohydrate accompanying the casings) are separated by distillation prior to color formation. The color is quite stable and sensitive in the range 1 to 10 μ g of formaldehyde per mL. The method may be applied to other products by following the procedure for total formaldehyde.

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

-
- a. Condenser.
 - b. Volumetric flasks: 1000 mL and 100 mL.
 - c. Distilling flask: 500 mL.
 - d. Volumetric pipettes: 1 mL.
 - e. Mohr pipette: 10 mL.
 - f. Glass beads.
 - g. Test tubes: 30 mL.
-

2. Instrumentation

Spectrophotometer suitable for reading at 570 nm.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List	<hr/>
--------------------------------------	-------

- | | |
|-------------|---|
| <div></div> | <div>a. Phosphoric acid (H_3PO_4) 85% ACS.</div> <div>b. Sulfuric acid solution—Add 300 mL of concentrated sulfuric acid to 150 mL of distilled water. Allow the mixture to cool.</div> <div>c. Chromotropic acid: Dissolve 1.13 g of 4, 5-dihydroxy-2, 7-naphthalene-disulfonic acid disodium salt (Practical) or 1.00 g of its acid, in 100 mL of distilled water, filter, and add 400 mL of the above sulfuric acid solution.</div> |
|-------------|---|
-

DETERMINATIVE METHOD

D. STANDARDS

Preparation of Standards

Formaldehyde.

- i. Dilute 5 mL reagent grade HCHO (38%) to 1 L with distilled water.
 - ii. Dilute 5 mL from step i to 1 L with distilled water. (1 mL = 10 μ g).
 - iii. Dilute 50 mL from step ii to 100 mL (1 mL = 5 μ g).
-

DETERMINATIVE METHOD

E. SAMPLE HANDLING

Sample Preparation

a. *Patty Paper* (free formaldehyde).

- i. Soak 10.0 g of patty paper in 200 mL of distilled water for 30 min.
- ii. Remove patty paper and transfer soak water to a 500 mL distilling flask quantitatively, using aliquots of distilled water.
- iii. Add 2 mL of 85% phosphoric acid and a few glass beads.
- iv. Distill into a 100 mL volumetric flask and dilute to volume.
- v. Proceed to section F.

b. *Sausage Casings*.

Prewash two 10.0 g samples of casing by allowing them to stand in 200 mL distilled water for 30 min.

i. *Total Formaldehyde*

- (a) Transfer a 10.0 g casing sample from the prewash to a 500 mL distilling flask containing 150 mL distilled water, 2 mL of 85% phosphoric acid, and glass beads.
- (b) When the sample is dispersed or broken up, distill into a 100 mL volumetric flask and dilute to volume with distilled water.
- (c) Proceed to section F.

ii. *Free Formaldehyde*

- (a) Transfer the second 10.0 g casing sample from prewash to a 500 mL distilling flask containing 100 mL distilled water.
 - (b) Stopper, agitate, hold at room temperature 30 min, and remove sample.
 - (c) Add 50 mL distilled water, 2 mL 85% phosphoric acid, and glass beads to the flask; distill into a 100 mL volumetric flask, and dilute to volume with distilled water.
 - (d) Proceed to section F.
-

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE

1. Determination

-
- a. Add 1 mL of the distillate, a distilled water blank, and standard solutions to separate clean test tubes.
 - b. Add 10 mL chromotropic acid solution rapidly, and mix.
 - c. Place tubes in steam or boiling water-bath protected from light, heat 30 min, remove, allow to cool, and transfer to cuvettes.
 - d. Measure absorbance at 570 nm using the distilled water blank to zero the spectrophotometer. Compare the optical density to a standard curve and calculate parts per million of formaldehyde.
-

2. Standard Curve

-
- a. Add 0 μg , 50 μg , 100 μg , 200 μg , 300 μg , 400 μg , and 500 μg respectively of formaldehyde from standard solution to 100 mL volumetric flasks and dilute to volume with distilled water.
 - b. Proceed as in section F. 1.
 - c. Prepare a curve plotting absorbance versus μg of formaldehyde per mL; read at 570 nm.
-

I. DETERMINATIVE METHOD

G. CALCULATIONS

1. Procedure

$$\text{Total or free formaldehyde (ppm)} = \frac{AB}{C}$$

A = μg formaldehyde per mL.

B = Final dilution volume in cuvette.

C = Sample weight (grams) in final volume.

Combined formaldehyde = total formaldehyde (ppm) – free formaldehyde (ppm)

2. Reference

Official Methods of Analysis of the Association of Official Analytical Chemists,
10th Edition, 27.031.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Determination of Formaldehyde in Sausage Casings and Patty Paper.		
2. Required Protective Equipment	Safety glasses, face shield, heat-resistant gloves, plastic gloves, lab coat.		
3. Procedure Steps	<u>Reagents</u>	<u>Hazards</u>	<u>Recommended Safe Procedures</u>
	a. Phosphoric acid 85%	Skin, eye irritation.	The preparation of these solutions and the color development should be performed in a fume hood.
	b. Formaldehyde standards	Flammable, corrosive skin, eye irritation.	
	c. Sulfuric acid	Skin, eye, respiratory irritation.	Protocols for diluting strong acids should be adhered to and use of protection equipment should be stressed.
	d. Chromotropic acid	Highly reactive toxin.	
4. Disposal Procedures	Aqueous reaction products	Corrosive and suspected toxic risk.	Dilute strong acids, cool, and flush down disposal sink with large amounts of water. Be sure that the area is well ventilated.

IRON AND CALCIUM BY ATOMIC ABSORPTION

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DETERMINATIVE METHOD

A. INTRODUCTION

Applicability

This procedure is applicable to the determination of iron and calcium in meat and poultry products.

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

-
- a. Erlenmeyer flask—125 mL.
 - b. Volumetric pipettes—5, 10, 20, 25, 30, 40, and 50 mL.
 - c. Volumetric flasks—100, 250, 1000 mL.
 - d. Hood—suitable for use with perchloric acid.
 - e. Blender.
-

2. Instrumentation

Atomic absorption spectrophotometer—establish parameters recommended by the instrument manufacturer.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and
Solution List

-
- a. Nitric acid.
 - b. Perchloric acid, 70%.
 - c. Lanthanum 5% stock solution. Wet 58.65 g of La_2O_3 with distilled water. Add 250 mL concentrated HCl very slowly until the material is dissolved. Dilute to 1 L with distilled water.
-

DETERMINATIVE METHOD

D. STANDARDS

Preparation of Standards

a. Calcium standards.

- i. Stock solution—dissolve 1.249 g of CaCO_3 in a minimum amount of 3N HCl and dilute to 1 L. Dilute 50 mL to 1 L ($25\mu\text{g/mL}$).
- ii. Working standards—0, 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 $\mu\text{g Ca/mL}$ containing 1% lanthanum—to 250 mL volumetric flasks add 0, 5, 10, 20, 30, 40, and 50 mL of calcium stock solution ($25\mu\text{g/mL}$). Add 50 mL of the 5% lanthanum stock solution and dilute to 250 mL. (Commercially prepared calcium standards are available.)

b. Iron standards.

- i. Stock solution—dissolve 1.000 g pure iron wire in approximately 30 mL 6N HCl with boiling. Cool, dilute 25 mL to 1 L ($25\mu\text{g/mL}$).
 - ii. Working standards—0, 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 $\mu\text{g Fe/mL}$ —use the dilutions recommended for calcium. *Omit the lanthanum solution.* (Commercially prepared standards are available.)
-

DETERMINATIVE METHOD

E. SAMPLE HANDLING

Sample Preparation

-
- a. Comminute sample in blender. Transfer 2 g of sample to a 125 mL Erlenmeyer flask.
 - b. Place three boiling beads into the flask. Add 15 mL of nitric acid to the sample and boil for a few minutes. This oxidizes the sample and reduces the possibility of explosions.
 - c. Remove sample from hot plate, cool, and add 5 mL of perchloric acid.
 - d. Place sample on the hot plate and boil until fumes of perchloric acid appear.
 - e. If sample chars at this point, remove from heat, cool, and add a few drops of nitric acid.
 - f. Place sample on the hot plate and continue heating until fumes of perchloric acid appear. Continue heating for 1 min and then remove from hot plate.

CAUTION: Never allow the sample to go to complete dryness.

- g. When sample has cooled, transfer the clear liquid to a 100 mL volumetric flask, add 5 mL of nitric acid, and make to volume. This is Solution A, which is used for the iron determination.
 - h. For the calcium determination, transfer a 10 mL aliquot of Solution A to 100 mL volumetric flask, add 20 mL of a 5% lanthanum solution, and make to volume. This solution is used for the calcium determination, using a 1% lanthanum solution in 5% (v/v) HCl to zero the instrument.
-

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE

Procedure

Read \geq standard solutions within the analytical range before and after each group of 6–12 samples. Flush burner with water between samples and reestablish 0 absorption point. Prepare a calibration curve from the average of each standard before and after sample group. Read concentration of sample from plot or if applicable read directly from instrument.

I. DETERMINATIVE METHOD

G. CALCULATIONS

1. Procedure

United States Recommended Daily Allowance (USRDA): (calcium) 1.0 g
(iron) 18 mg

$$\text{mg/serving} = \frac{\text{ppm}}{\text{Wt} \times 1000} \times F$$

$$\% \text{ USRDA} = \frac{\text{mg/serving}}{\text{RDA}} \times 100$$

ppm = concentration ($\mu\text{g/mL}$) of element

Wt = weight of sample in final dilution (g/mL)

1000 = microgram per milligram

F = serving size converted to the appropriate dimensions (i.e., ounces to grams, etc.)

2. Reference

Official Methods of Analysis of the Association of Official Analytical Chemists, 15th Edition.

IRON/SPECTROPHOTOMETRIC

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DETERMINATIVE METHOD

A. INTRODUCTION

Applicability

This procedure is applicable to the determination of iron in meat or poultry products.

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

-
- a. Vycor crucible: 50 mL.
 - b. Volumetric flasks: 25, 100, 500, 1000 mL.
 - c. Volumetric pipettes: 2, 5, 10, 20, 30, 40 mL.
 - d. Funnel.
 - e. Filter paper.
-

2. Instrumentation

Spectrophotometer suitable for reading at 510 nm.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and
Solution List

-
- a. Hydrochloric acid: Concentrated, reagent grade.
 - b. Hydroxylamine hydrochloride: Dissolve 10.0 g $\text{NH}_2\text{OH}\cdot\text{HCl}$ in H_2O and dilute to 100 mL.
 - c. Buffer solution: Dissolve 8.3 g anhydrous NaOAc (dried at 100°C) in H_2O , add 12 mL glacial acetic acid, and dilute to 100 mL.
 - d. Dipyridyl solution: Dissolve 0.100 g alpha, alpha-dipyridyl in H_2O and dilute to 100 mL (stable several weeks in refrigerator).
-

DETERMINATIVE METHOD

D. STANDARDS

Preparation of Standards

-
- a. Stock iron solution: Dissolve 3.512 g $\text{Fe}(\text{NH}_4)_2 \cdot (\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ in water, add 2 drops HCl, and dilute to 500 mL.
 - b. Working iron standard: Dilute 10 mL standard iron solution to 1 L (1 mL = 0.01 mg Fe).
-

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE

-
- | | |
|-------------------|--|
| 1. Standard Curve | Add 0, 10, 20, 30, and 40 mL of the working standard plus 2 mL HCl in 100 mL volumetric flask, dilute to volume. Take 10 mL aliquots and proceed as outlined in section F.2.f and g. Plot absorbance vs. mg Fe/mL. |
|-------------------|--|
-
- | | |
|------------------|--|
| 2. Determination | <ol style="list-style-type: none">a. Weigh 10.0 g ground sample into a 50 mL vycor crucible and spread in a thin layer on inside walls of the crucible.b. Dry in a 125° C oven and then ash in muffle furnace at 550-600° C. Continue ashing until practically carbon-free.c. Cool, moisten ash with distilled water, add 5 mL HCl, and evaporate to dryness on steam bath.d. Take up residue in 2.0 mL HCl, heating 5 min on steam bath, and rinse through filter into 100 mL volumetric flask. Dilute to volume with distilled water.e. Pipette 10 mL aliquot into a 25 mL volumetric flask, set up a reagent blank with 10 mL distilled water plus 2 mL HCl and one or two standards using 10 mL aliquots of iron standards used in preparing working standard curve.f. Add 1 mL $\text{NH}_2\text{OH}\cdot\text{HCl}$ solution, mix thoroughly by swirling, add 5 mL buffer solution followed by 2 mL dipyrldyl solution, and dilute to volume.g. Transfer solutions to cuvettes and read optical density in spectrophotometer at 510 nm. |
|------------------|--|
-

DETERMINATIVE METHOD

G. CALCULATIONS

1. Procedure

United States Recommended Daily Allowance (USRDA) = 18 mg

$$\text{mg/serving} = \frac{A \cdot B \cdot F}{Wt}$$

A = concentration of iron (mg/mL) from standard curve

B = final volume (mL)

F = serving size converted to the appropriate dimensions
(i.e., ounces to grams, etc.)

Wt = weight (g) of sample aliquot in final dilution

2. Reference

Official Methods of Analysis of the Association of Official Analytical Chemists,
15th Edition.

FLUORIDE IN MECHANICALLY SEPARATED SPECIES

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DETERMINATIVE METHOD

A. INTRODUCTION

Theory

Mechanically Separated Species samples are defatted with petroleum ether. Disodium EDTA is added to complex calcium, and a total ionic strength adjustor is added to complex interfering ions such as aluminum and iron and to provide a constant background ionic strength, decomplex fluoride, and adjust solution pH. Final sample solution is then measured on a millivolt meter with fluoride-specific ion and reference electrodes.

DETERMINATIVE METHOD

B. EQUIPMENT

Apparatus

-
- a. pH/mv meter—Orion Model 701, or equivalent.
 - b. Fluoride ion electrode—Orion No. 94-09, or equivalent.
 - c. Reference electrode—Single-junction, Orion No. 90-01, or equivalent.
 - d. Tissue blender—Model SDT Tissuemizer, Tekmar Co., Cincinnati, OH 45222, or equivalent.
 - e. Centrifuge—Equipped with accessories for 250 mL centrifuge bottles, IEC PR-600, or equivalent.
 - f. Centrifuge bottles—250 mL capacity, Corning No. 1280, or equivalent.
 - g. Silicone stoppers—No. 6, Thomas No. 8747-E65, or equivalent.
 - h. Constant-temperature water bath with cover—Blue M Model MW1120A, or equivalent.
 - i. Polyethylene bottles—16 and 32 oz Thomas No. 1704-C38 and 1704-C48, or equivalent.
 - j. Polypropylene beakers—100 and 250 mL, Thomas No. 1541-F35 and 1541-F45, or equivalent.
 - k. Volumetric flasks—1 L and 100 mL, Thomas No. 4997-B70 and 4997-B47, or equivalent.
-

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List	<div data-bbox="501 420 1481 667" data-label="List-Group"><ul style="list-style-type: none">a. Petroleum ether—distilled in glass, Burdick and Jackson, Muskegon, MI 49442, or equivalent.b. Disodium ethylenediamine tetraacetic acid—5% w/v, in distilled water.c. Total ionic strength adjustor (TISAB)—Dissolve 116 g NaCl and 0.6 g sodium citrate in 114 mL glacial acetic acid and 1000 mL distilled water. Add 5N NaOH until pH is 5.0-5.5. Cool to room temperature and dilute to 2 L with distilled water.</div>
--------------------------------------	--

DETERMINATIVE METHOD

D. STANDARDS

1. Preparation of Standards

a. Standard solutions (prepare in volumetric flasks).

- i. Stock Solution A—Carefully weigh 2.2101 g NaF and dilute to 1 L with distilled water (1000 ppm F^-).
- ii. Stock Solution B—Dilute 10 mL stock solution A to 1 L with distilled water (10 ppm F^-).
- iii. Stock Solution C—Dilute 10 mL stock solution B to 100 mL with distilled water (1.0 ppm F^-).

b. Working Standards (in volumetric flasks).

- i. Fluoride ion concentration, (F^-) = 0.03 ppm. To 3 mL stock solution C, add 20 mL TISAB and dilute to 100 mL with distilled water.
- ii. (F^-) = 0.05 ppm. To 5 mL stock solution C, add 20 mL TISAB and dilute to 100 mL with distilled water.
- iii. (F^-) = 0.10 ppm. To 10 mL stock solution C, add 20 mL TISAB and dilute to 100 mL with distilled water.
- iv. (F^-) = 0.50 ppm. To 50 mL stock solution C, add 20 mL TISAB and dilute to 100 mL distilled water.
- v. (F^-) = 1.00 ppm. To 10 mL stock solution B, add 20 mL TISAB and dilute to 100 mL with distilled water.
- vi. (F^-) = 10.00 ppm. To 1 mL stock solution A, add 20 mL TISAB and dilute to 100 mL with distilled water.

NOTE: Higher concentrated standards may be prepared if necessary. Linear range for millivolt readings extends beyond, but not below, concentrations described above.

2. Storage Conditions

Store in polyethylene bottles.

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE

1. Determination

-
- a. Weigh 10 g sample into 16 oz polyethylene bottle.
 - b. Run a tissue blank and a recovery in parallel with sample(s). Add 10.0 mL of 10.0 ppm F^- standard solution to 10 g blank tissue for 10 ppm F^- fortification.
 - c. Extract fat twice with petroleum ether as follows: Add 100 mL petroleum ether to each sample, blank, and recovery. Break up lumps with plastic stirring rod. Stopper with silicone stopper and shake vigorously for 30 sec. Centrifuge for 10 min at 2,500 rpm. Decant and discard ether layer, being careful not to include any aqueous phase. Repeat extraction.
 - d. Place sample bottles in water bath and carefully evaporate any residual ether by gradually raising the bath temperature to 100° C to prevent spattering.
 - e. Remove samples immediately, cool, and add 50 mL TISAB solution and 50 mL 5% disodium EDTA. Mix with Tissumizer until homogenous.
 - f. Place sample bottles in covered constant-temperature water bath that has attained nearly 100° C. (Salt may be added to bath to increase boiling point.)
 - g. Place thermometer into one sample bottle until temperature of sample reaches 90° C. Remove cover and leave samples in bath for 1 hr. (Samples will reach an equilibrium temperature of approximately 94° C.) Remove samples and let cool.
 - h. Break up lumps with plastic stirring rod.
 - i. Transfer samples to 250 mL graduated cylinders. Rinse bottle several times into cylinder and dilute samples to 250 mL with distilled water.
 - j. Stopper and shake thoroughly.
 - k. Filter sample solutions through fluted filter paper into polyethylene bottles. Do not use glass bottles.
 - l. Pour about 100 mL sample solution into polypropylene beaker and with constant stirring, insert electrodes, and record mv reading at equilibrium.
-

2. Preparation of Standard Curve

-
- a. According to manufacturer's instructions, verify that pH/mv meter reads from -200 to +200 mv.
 - b. Place electrodes in 100 mL polypropylene beaker containing 10.00 ppm working standard on a magnetic stirrer, and stir. Set the meter function switch to read expanded millivolts and turn calibration control to read 000.0 mv.
 - c. Rinse electrodes, blot dry, and place in 1.00 ppm F^- standard. Stirring continuously, wait for reading to stabilize. Stabilization is indicated by no more than 0.5 mv change over a 5 min period. Waiting period increases with decreasing concentration.
-

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE (Continued)

-
- d. Repeat step c, above, with rest of the standards in order of decreasing concentration.
 - e. On standard 2-cycle semi-log paper, plot mv versus concentration, with mv on the horizontal axis and concentration on the vertical (log) axis. Three-cycle paper can be used if higher concentrated standards are not required.
-

DETERMINATIVE METHOD

G. CALCULATIONS

1. Procedure

Compare mv sample reading to standard curve to obtain ppm F⁻ in sample solution.

$$\text{ppm F}^- \text{ in sample} = \frac{A B}{C}$$

where: A = ppm F⁻ in sample solution

B = mL final dilution

C = g sample

If procedure is followed as written, $\text{ppm F}^- = \frac{250A}{10} = 25A$

Note: Specific ion and pH meters equipped with direct concentration readout via microprocessor may be used.

2. Reference

Dolan, et al: *J. Assoc. Off. Anal. Chem.* (Vol. 61, No. 4, 1978, p 982).

INTERNAL COOKING TEMPERATURE

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DETERMINATIVE METHOD

A. INTRODUCTION

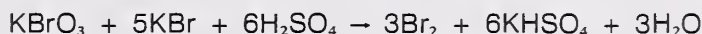
1. Theory

The enzyme (protein) acid phosphatase is denatured by heat. The activity of the phosphatase left after cooking is expressed as the amount of phenol formed when the sample is allowed to act upon the substrate disodium phenylphosphate for a constant time, at a constant temperature, and a fixed pH. The phenol produced is reacted with 2,6,-dibromoquinone chlorimide, to yield indophenol blue. The absorbance of the blue color formed is measured spectrophotometrically at 610 nm.

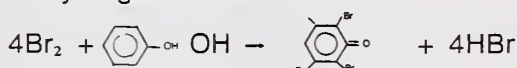
This method involves incubation of weighed samples with sodium phenyl phosphate in a constant temperature bath. Active phosphatase cleans this into phenol and sodium phosphate.

The mechanism for the standardization of the stock phenol solution is shown by the following equations:

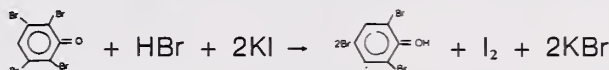
Potassium bromate + potassium bromide + $\text{H}_2\text{SO}_4 \rightarrow$ bromine
+ potassium chloride + water



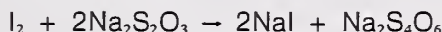
Phenol + bromine \rightarrow tetrabromophenol hypobromite
+ hydrogen bromide



Tetrabromophenol hypobromite + hydrogen bromide
+ potassium iodide \rightarrow tribromophenol + potassium bromide
+ iodine



The iodine is titrated with thiosulfate and the amount of phenol is calculated.



Each mL 0.1N potassium bromate = 0.001569 g phenol.

2. Applicability

The regulations require that all processed pork products be cooked to a temperature high enough to kill trichinae. In addition, APHIS Veterinary Services requires an internal cooking temperature of 156° F on imported pork products, from certain countries, to kill the foot-and-mouth virus and other exotic viruses. The ICT1 and ICT2 methods are used to determine the maximum internal cooking temperature reached in the processing of a meat product.

The coagulation test (method ICT2) is suitable as a screening method for use on all meat products for temperatures below 150° F. Above 150° F, the method is not accurate and this phosphatase procedure should be used, but only on canned picnics and canned hams, received either in the can or in a hard frozen condition.

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

NOTE: Do not use plastic labware.

- a. 50 mL glass-stoppered centrifuge tubes (Pyrex 8424 or equivalent).
- b. 15 mL test tubes.
- c. Constant temperature waterbath (37° C).
- d. 500 mL Erlenmeyer flasks.
- e. pH meter.

2. Instrumentation

-
- a. Spectrophotometer—suitable for reading at 610 nm.
 - b. Pipettes—various sizes.
 - c. Stopwatch.
 - d. Syringe—100 μ L.
-

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

- a. Citrate buffer, pH 6.5 ± 0.1 : Dissolve 41.64 g trisodium citrate and 1.765 g citric acid in distilled water and dilute to 3 L. Adjust to pH 6.5 with pH meter. Preserve with 3 mL toluene and store in refrigerator.
- b. 50% trichloroacetic acid: Dissolve 500 g TCA in distilled water and dilute to 1 L.
- c. 20% trichloroacetic acid: Dilute 200 mL 50% TCA to 500 mL with distilled water.
- d. 5% trichloroacetic acid: Dilute 100 mL 50% TCA to 1 L with distilled water.
- e. Sodium carbonate 0.5M: Dissolve 53 g anhydrous sodium carbonate in distilled water and dilute to 1 L.
- f. 2,6-dibromoquinone chlorimide (make fresh daily): Dissolve 40 mg 2,6-dibromoquinone chlorimide in 10 mL absolute alcohol. (Store reagent itself in a brown bottle in a desiccator).
- g. Disodium phenyl phosphate 0.01M: Dissolve 0.436 g disodium phenyl phosphate in distilled water and dilute to 200 mL. (Prepare immediately before use.)
- h. Stock phenol solution: Dissolve 1.000 g phenol in distilled water and dilute to 1 L.
- i. Working phenol solution: Transfer 5 mL of stock phenol solution (item h above) to 1 L volumetric flask. Add 100 mL 50% TCA and dilute to volume with distilled water. Shake well.

The following reagents are for standardization of stock phenol solution:

- j. Starch indicator (prepared solutions can be purchased): Mix 1 g soluble starch with 5 mL water. Add to 95 mL boiling water. Mix, cool, filter, and add 0.01 g HgI_2 .
- k. Sodium thiosulfate 0.1N: Dissolve 25 g sodium thiosulfate pentahydrate and 0.2 g sodium carbonate and dilute to 1 L with freshly boiled water.
- l. Potassium iodide 10%: Dissolve 5 g KI and dilute to 50 mL with distilled water.
- m. Sulfuric acid 2N: Dilute 5.6 mL concentrated H_2SO_4 to 100 mL with distilled water.
- n. Hydrochloric acid 2N: Dilute 17.8 mL concentrated HCl to 100 mL with distilled water.
- o. Potassium bromide: Reagent grade.
- p. Potassium bromate 0.1N: Dissolve 2.783 g potassium bromate in distilled water and dilute to 1 L.

DETERMINATIVE METHOD

D. STANDARDS

1. Preparation of Standards

-
- a. Pipet duplicate aliquots of 0.0 mL, 0.5 mL, 1.0 mL and 2.0 mL of working phenol solution into 15 mL test tubes (8 tubes total).
 - b. Pipet 5.0 mL, 4.5 mL, 4.0, and 3.0 mL respectively, of 5% TCA, making each tube equal in volume (5 mL).
 - c. Add 5.0 mL 0.5M sodium carbonate.
 - d. Pipet 0.1 mL 2,6-dibromoquinone chlorimide into each tube.
 - e. Swirl and develop color for at least 30 min in the dark.
 - f. Measure the absorbance of each tube at 610 nm, using 1 cm cells and water as reference for setting spectrophotometer at 100% transmission.
-

2. Determination of Sodium Thiosulfate Factor

- a. Add 2 g KI, 25 mL 0.1N potassium bromate and 20 mL 2N HCl in an 500 mL Erlenmeyer flask.
 - b. Let stand 15 min in the dark.
 - c. Slowly add 150 mL distilled water.
 - d. With steady swirling or on magnetic stirrer, titrate with 0.1N sodium thiosulfate until the blue color disappears, using 1 mL of starch solution as indicator.
-

3. Determination of Phenol Concentration in Stock Phenol Solution

- a. Pipet 50.0 mL stock phenol solution into glass-stoppered 500 mL Erlenmeyer flask.
 - b. Pipet 50.0 mL 0.1N potassium bromate solution into the flask.
 - c. Add 2.0 g potassium bromide.
 - d. When the latter has dissolved, add 20 mL 2N H₂SO₄. Mix.
 - e. Let stoppered solution sit for 15 min in the dark.
 - f. Carefully pipet 10 mL 10% KI into mixture, removing stopper as little as possible.
 - g. Shake well and let sit 15 min in the dark.
 - h. Titrate with 0.1N sodium thiosulfate after rinsing stopper and sides of flask with distilled water, adding 1 mL starch indicator. End point is indicated by the absence of blue color from the gel particles. A bright light and white background may be necessary.
-

DETERMINATIVE METHOD

F. ANALYTICAL DETERMINATION

Determination

-
- a. Weigh 2.50 g sample into each of four glass-stoppered 50 mL test tubes (A, B, C and D). Tubes A and B are to be used for duplicated determinations. Tubes C and D are duplicate control samples.
 - b. Pipet 10 mL citrate buffer into each tube.
 - c. Pipet 5 mL 20% TCA into control samples C and D only.
 - d. Stopper and shake well.
 - e. Place all tubes in a water bath at $37.0^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for 10 min before proceeding.
 - f. Pipet 5 mL disodium phenyl phosphate solution into each tube in turn at exactly 60-sec intervals using a stopwatch.
 - g. Shake all tubes at 10-min intervals.
 - h. After exactly 60 min, by stopwatch, pipet 5 mL 20% TCA to each tube in turn at 60 sec intervals *except tubes C and D*. (Tube A at 60 min, tube B at 61 min, etc.).
 - i. Remove each tube from water bath after addition of TCA, shake well and filter twice through Whatman 2V filter paper. (Filtrates may be stored at 4°C if needed.)
 - j. Pipet 3 mL clear filtrates into clean test tubes.
 - k. Pipet 3 mL sodium carbonate 0.5M, into each tube. Swirl to mix.
 - l. Add 100 μL 2,6-dibromoquinone chlorimide solution into each tube, using an 100 μL syringe. Mix well by swirling.
 - m. Develop color in the dark for at least 30 min (not overnight). If tube C or D is blue, contamination has occurred. Begin test again, using new 2.5 g samples and clean labware.
 - n. Read absorbance of each solution at 610 nm using 1 cm cells and water as reference for setting the spectrophotometer at 100% transmission. Spectrophotometer should be calibrated with a holmium oxide crystal.
-

DETERMINATIVE METHOD

G. CALCULATIONS

1. Procedure

$$a. A = 0.1N \text{ potassium bromate factor} = \frac{\text{g potassium bromate weighed}}{2.783}$$

$$B = 0.1N \text{ sodium thiosulfate factor} = \frac{25.00 \times A}{\text{mL C}}$$

C = mL thiosulfate titrated in section D.2, step d.

$$b. \% \text{ Phenol} = \frac{[(50)(A) - (B)(D)] 0.1569}{50}$$

D = mL thiosulfate titrated in section D.3, step h.

$$c. X = \text{mg phenol/100 mL stock solution}$$

$$d. Y = \frac{\mu\text{moles phenol/1000 mL working phenol solution}}{\text{Molecular wt of phenol}} = \frac{(X)(5)(1)}{94.11}$$

$$e. \text{Extinction} = \frac{(\text{absorbance tube A} + \text{absorbance tube B})}{2} - \frac{(\text{absorbance tube C} + \text{absorbance tube D})}{2}$$

Read absorbance to the nearest 0.001 absorbance unit.

After correcting for the blank (abs of the 0.0 mL standard substituted in the above formula in place of abs tubes C and D), the extinction values of the standard solutions are treated to determine the standard factor F.

$$f. F \text{ 0.5 standard} = \frac{0.5Y}{\text{extinction}} = \mu\text{mol phenol/extinction unit}$$

$$g. F \text{ 1.0 standard} = \frac{Y}{\text{extinction}} = \mu\text{mol phenol/extinction unit}$$

$$h. F \text{ 2.0 standard} = \frac{2Y}{\text{extinction}} = \mu\text{moles phenol/extinction unit}$$

$$i. F = \frac{F \text{ 0.5 standard} + F \text{ 1.0 standard} + F \text{ 2.0 standard}}{3}$$

DETERMINATIVE METHOD

G. CALCULATIONS (Continued)

To evaluate the phosphatase activity of the sample:

$$j. \quad EF' = \mu\text{mol phenol}/1000 \text{ g sample} = \frac{(F)(\text{sample extinction})(1000)}{62.5}$$

NOTE: 62.5 = the dilution factor used to convert g/mL to micro moles phenol, 1000 g sample.

$$\frac{2.5 \text{ g}}{20 \text{ mL}} \times \frac{3 \text{ mL}}{6 \text{ mL}} \times 1000$$

$$k. \quad ^\circ\text{C internal cooking temperature} = 77.3985 - (5.7109)(\text{Log } EF')$$

$$l. \quad ^\circ\text{F} = \frac{9}{5} ^\circ\text{C} + 32$$

The formula used to calculate the internal temperature was derived empirically. It may be necessary to redetermine the formula if processing procedures are changed.

NOTE: 1. Correction for products with salt content higher than 3.55%.
Subtract 0.95° F for each 1% above 3.55%.

2. Repeat analysis if the following criteria are not met.

Abs of tubes A & B between	Acceptable abs difference between tubes A and B
0.35 to 0.50	0.035
0.20 to 0.34	0.028
0.10 to 0.19	0.020
Less than 0.10	0.016

2. Reference

Lind. J. Determination of Activity of Acid Phosphatase in Canned Hams, Danish Meat Products, Laboratory, The Royal Veterinary and Agriculture College, September 23, 1965.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Determination of Internal Cooking Temperature (Phosphatase).		
2. Required Protective Equipment	Safety glasses, plastic gloves, lab coat.		
3. Procedure Steps		<u>Hazards</u>	<u>Recommended Safe Procedures</u>
	Trichloroacetic acid	Eye, skin, and respiratory irritation.	Prepare and dispense in an efficient fume hood.
	2, 6-dibromoquinone chlorimide	Explosive at 120° C.	Store in refrigerator. Keep away from any heat source when using in lab.
	Phenol solutions	Highly toxic and suspected carcinogen. Rapidly absorbed through the skin.	Protective gear must be stressed. Work in cool, well-ventilated area.
4. Disposal Procedures	Colorimetric reaction solution	Mild irritant.	Flush into disposal sink with large quantities of water.
	Samples found to be undercooked	Spread of exotic animal diseases.	Entire sample must be autoclaved or incinerated.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standard

<i>Procedure</i>	<i>Analytical Range (°)</i>	<i>Repeatability % CV</i>	<i>Reproducibility % CV</i>
Maximum Internal Temperature	~145° F or ~62.83° C ~160° F or ~71.17° C	(±3° F)	(±5° F)

2. Critical Control Points and Specifications

<i>Record</i>	<i>Acceptable Control</i>
Labware	Do not use phenolic plastic labware.
Citrate buffer	6.5 ± 0.1 pH. Check with pH meter, calibrated at 6 or 7 pH, just prior to use.
Alcohol for 2,6-dibromoquinone chlorimide solution	Must be <i>absolute</i> .
Potassium bromate standardization	Standardize as in AOAC, 14 Ed., Sec 50.005, 50.006, 50.020, and 50.021.
Sample size	2.50 ± 0.02 g.
Water bath	37.0° C ± 0.5° C. Bring tubes to temperature before proceeding to next step.
Timed intervals	<i>Exactly</i> 60 sec, by stopwatch, for reagent additions from one tube to the next, and exactly 60 min, by stopwatch, for each tube in sequence, except C and D, for addition of TCA reagent.
Volume of BQC	Exactly 100 µL.
Color development	Must be in the dark for not less than 30 min. An hour is acceptable, but not overnight.
Spectrophotometer	Calibrated, capable of using 1 cm cells. Variable wavelength capability. Use distilled water as a reference for setting "0" absorbance.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

	<i>Record</i>	<i>Acceptable Control</i>
	Standards	Duplicate within ± 0.010 absorbance units.
	Sample tubes A & B	Duplicate within: 0.035 absorbance units for reading 0.35-0.50 0.028 absorbance units for reading 0.20-0.34 0.020 absorbance units for reading 0.10-0.19 0.016 absorbance units for reading < 0.10
	Calculations	Recheck.
3. Readiness To Perform	a. Familiarization.	
	i. Phase I: Standards—	
	(a) Prepare standards and measure absorbance.	
	(b) Determine sodium thiosulfate factor.	
	(c) Determine phenol concentration in stock phenol solution.	
	ii. Phase II: Fortified samples	
	iii. Phase III: Check samples for analyst accreditation.	
	b. Acceptability criteria.	
	See section J.1 above.	
4. Intralaboratory Check Samples	a. System, minimum contents.	
	i. Frequency: 1 per week not to exceed 20% of sample.	
	ii. Blind samples or random replicates chosen by supervisor after initial analysis.	
	iii. Records are to be maintained by analyst and reviewed by the supervisor and Laboratory QA Officer.	
	b. Acceptability criteria.	
	If unacceptable values are obtained, then:	
	i. Stop all official analyses for that analyst.	
	ii. Investigate and identify probable cause.	
	iii. Take corrective action.	
	iv. Repeat Phase III of section J.3 if cause was analyst-related.	

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

**5. Sample
Acceptability
and Stability**

- a. Matrix: Canned picnics and canned hams
 - b. Sample receipt size, minimum: 1 lb
 - c. Condition upon receipt: In can or hard frozen
 - d. Sample storage:
 - i. Time: One month
 - ii. Condition: Frozen
-



INTERNAL COOKING TEMPERATURE/COAGULATION

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DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory

Soluble proteins are extracted with a 0.9% saline (NaCl) solution and subjected to heat, causing a cloudiness or turbidity in the extract because of coagulated proteins. The temperature at which the first sign of cloudiness appears is the maximum internal cooking temperature. This procedure is empirical and must be followed as written.

2. Applicability

This procedure is applicable to both beef and pork products below 150° F that do not contain any organ meats or nonmeat proteins.

DETERMINATIVE METHOD

B. EQUIPMENT

Apparatus

-
- a. Gooch crucible.
 - b. Celite 545.
 - c. Buchner funnel.
 - d. Filtering flask with tubulation.
 - e. Filter paper—suitable for use in Buchner funnel.
 - f. Test tube—50 mL.
 - g. Water bath—consisting of 1 to 2 L beaker with a stirring apparatus and clamps or rack for holding test tubes.
 - h. Bunsen burner or equivalent.
 - i. Two identical thermometers.
 - j. 250 mL beaker.
-

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List	<p>0.9% saline (NaCl) solution: Dissolve 9.0 g of sodium chloride in 200 mL of H₂O. Make to 1 L, mix thoroughly.</p>
------------------------------	---

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE

1. Determination

-
- a. Weigh 50 g of ground sample into a 250 mL beaker.
 - b. Add 100 mL of saline (NaCl) solution; thoroughly mix and allow to stand for 20 min.
 - c. Filter the mixture through a filter paper on a Buchner funnel using vacuum.
 - d. Filter the filtrate a second time using a Gooch crucible and a coarse filter paper with a Celite 545 pad of appropriate thickness.
 - e. The second filtrate should be nearly clear; if not, repeat step d. NOTE: If filtrate is too clear, all protein may have been removed.
 - f. The filtrate is divided into two test tubes. One is the control, the other the test portion.
 - g. The test sample is clamped into position in the water bath and a thermometer is suspended in the filtrate. A second thermometer is suspended in the water.
 - h. Apply heat to the water being careful to maintain a difference $\leq 1.5^{\circ}$ F between the water temperature and the temperature of the filtrate.
 - i. The temperature at which the first sign of cloudiness appears compared to the control portion, is the maximum internal cooking temperature. Continue to heat up to 156° F. If a definite cloudiness does not appear, add ca. 1 mL 20% phosphotungstic acid to confirm presence of protein. If no cloudiness appears, start over.

NOTE: For rapid screening, the filtrate may be immersed in a water bath at 156° F. Those samples that appear to cloud may be removed and a fresh aliquot of the same samples treated to slower temperature rise to determine the maximum cooking temperature, is used.

2. Reference

USDA, Food Safety and Inspection Service, Chemistry Division, unpublished method.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Determination of Internal Cooking Temperature (Coagulation).
2. Required Protective Equipment	Safety glasses, plastic gloves, lab coat.
3. Procedure Steps	No unusual safety hazards in this method.
4. Disposal Procedures	Good hygienic practice should be used in disposing of the meat that has been extracted.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standard

<i>Procedure</i>	<i>Analytical Range</i>	<i>Repeatability % CV</i>	<i>Reproducibility % CV</i>
Maximum Internal Temperature	< 150° F	(±3° F)	(±5° F)

2. Critical Control Points and Specifications

<i>Record</i>	<i>Acceptable Control</i>
Sample size	50.0 g ± 0.1 g.
Volume of saline solution	100 ± 1 mL.
Filter paper filtration	Must be done. Coarse paper may be used.
Gooch filter media	Must be Celite 545.
Heat apparatus	Hang tube containing meat extract so that surface of extract is below surface of water bath and tube is not touching side or bottom of water bath. Hang calibrated thermometers in the meat extract and the water bath so that they are not touching the tube or the beaker. Thermometers should be alike in divisions and temperature ranges. Entire bulb must be submerged.
Heating rate	Start at room temperature. Increase temperature at rate of 1.0-1.5° F per min, keeping both thermometers within 1.5° F of one another. Stir bath and extract to ensure equilibrium of temperature.
Maximum internal cooking temperature	Record thermometer reading on meat extract at <i>first</i> sign of cloudiness, <i>not</i> color change.
Reporting	See appropriate memoranda and directives. Rerun all noncomplying samples before reporting.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

3. Readiness To Perform

-
- a. Familiarization.
 - i. Phase I: Standards—NA.
 - ii. Phase II: Replicates from previously analyzed samples.
 - iii. Phase III: Check samples for analyst accreditation.
 - b. Acceptability criteria.

See section J.1 above.
-

4. Intralaboratory Check Samples

-
- a. System, minimum contents.
 - i. Frequency: 1 per week, not to exceed 20% of samples.
 - ii. Blind samples or random replicates chosen by supervisor after initial analysis.
 - iii. Records are to be maintained by analyst and reviewed by the supervisor and Laboratory QA Officer.
 - b. Acceptability criteria.

If unacceptable values are obtained, then:

 - i. Stop all official analyses for that analyst.
 - ii. Investigate and identify probable cause.
 - iii. Take corrective action.
 - iv. Repeat Phase III of section J.3 if cause was analyst-related.
-

5. Sample Acceptability and Stability

-
- a. Matrix: Beef or pork.
 - b. Sample receipt size, minimum: 1 lb.
 - c. Condition upon receipt: Frozen preferred, but evidence of ice crystals acceptable.
 - d. Sample storage:
 - i. Time: 3 months.
 - ii. Condition: 0° C.
-

MOISTURE ABSORPTION IN GIBLET WRAP

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DETERMINATIVE METHOD

A. INTRODUCTION

Theory

The amount of moisture absorbed by gilet paper is such that (1) the wet weight of the paper shall not exceed 90 lbs per ream, and (2) the amount of moisture absorbed shall not exceed 200% of the dry weight of the paper.

MGP
May, 1993

DETERMINATIVE METHOD

B. EQUIPMENT

Apparatus

-
- a. 2 x 4" stainless steel die.
 - b. Analytical balance.
 - c. Scalpel.
-

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE

Procedure

-
- a. Select ten representative sheets of paper from the sample.
 - b. Using the 2 × 4" die, cut 2 X 4" strips from each sheet.
 - c. Determine the dry weight of each 2 × 4" strip using an analytical balance.
 - d. Immerse each strip in a beaker of water for 1 min.
 - e. Remove the strip from the beaker and shake lightly to remove excess water.
 - f. Fold the strip lengthwise twice to reduce evaporation. *Do Not Squeeze.*
 - g. Weigh each folded strip.
-

DETERMINATIVE METHOD

G. CALCULATIONS

1. Procedure

-
- a. Determine the average weight of the dry strips.
 - b. Determine the average weight of the wet strips.
 - c. Percent moisture absorbed = $\frac{A - B}{B} \times 100$

A = average weight of wet strips.

B = average weight of dry strips.

d. Wet weigh per ream = $\left(\frac{\text{Percent moisture absorbed (C)}}{100} \right) + C$

Where C = dry weight per ream of paper being tested. Obtained from:

If B equals	then C equals
0.2856 g	34 lbs
0.2771	33
0.2687	32
0.2604	31
0.2520	30
0.2436	29
0.2352	28
0.2268	27

(Extrapolate if necessary).

2. Reference

Technical Association of the Pulp and Paper Industry (T.A.P.P.I.), Standards T-410 and T-402.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Determination of Moisture Absorption of Giblet Wrap.
2. Required Protective Equipment	Safety glasses, plastic gloves, lab coat.
3. Procedure Steps	No unusual safety hazards in this method.
4. Disposal Procedures	No meat or chemicals used in this method.

MOISTURE

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DETERMINATIVE METHOD

A. INTRODUCTION

Theory

In this determination, a weighed sample is heated, cooled, and then reweighed.
The loss in weight is calculated as moisture content.

DETERMINATIVE METHOD

B. EQUIPMENT

Apparatus

-
- a. Covered aluminum dish. At least 50 mm diameter and not greater than 40 mm deep, containing a paddle.
 - b. Mechanical convection oven, preferably one equipped with a booster heater.
-

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE

Procedure

-
- a. Accurately weigh sample (representing approximately 2 g of dry material) into an aluminum dish.
 - i. Weigh the sample as rapidly as possible to minimize loss of moisture.
 - ii. The weight of the pan should include the paddle, which is used in spreading the sample across the bottom of the pan, thereby presenting a greater sample surface area, which is beneficial to moisture removal.
 - iii. If the sample is relatively dry when received, a small quantity of distilled water may be added to the pan only *after* the sample weight is obtained. This quantity of water will be helpful in spreading the sample across the bottom of the pan, and will introduce no error since it will be evaporated when the sample is oven-dried.
 - b. Dry, with cover removed, for 16-18 hr at 100-102° C, or for 4 hr at 125° C, in mechanical convection oven.

Do not overload the drying oven or sample may be insufficiently dried and give low results. Drying time will start when the original temperature has been reached. Use the oven's booster heater, if the oven is so equipped, to minimize this recovery time.

DETERMINATIVE METHOD

G. CALCULATIONS

1. Procedure

$$\text{Percent} = \frac{100(B - C)}{A}$$

A = sample weight

B = weight of dish + sample prior to drying

C = weight of dish + sample after drying

NOTE: If laboratory is not air-conditioned, and humidity is high, it is advisable to desiccate dishes prior to the initial and final weighings.

2. Reference

Official Methods of Analysis of the Association of Official Analytical Chemists, 15th Edition, 950.46.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Moisture Determination.
2. Required Protective Equipment	Safety glasses, heat-resistant gloves, lab coat.
3. Procedure Steps	No unusual safety hazards in this method.
4. Disposal Procedures	Use good hygienic practice in disposing of dried meat samples.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standard

<i>Compound</i>	<i>Analytical Range (%)</i>	<i>Repeatability % CV</i>	<i>Reproducibility % CV</i>
Moisture	‡	<0.46*	<0.65*

‡ Limit may vary due to sample and aliquot sizes and sample type.
* Standard deviation.

2. Critical Control Points and Specifications

<i>Record</i>	<i>Acceptable Control</i>
Sample size	3-6 g (representing about 2 g dry material).
Dish size	≥ 50 mm diameter; ≤ 40 mm deep; with cover.
Oven temperature	101° ± 1° C (for 16-18 hrs after oven reaches temperature) 125° ± 1° C (for 4 hrs ± 10 min after oven reaches temperature) in mechanical convection, forced-air oven. Check with calibrated thermometer.
Oven overloading	No dishes touching and not placed on solid tray—proper air circulation required.
Oven recovery	Return to temperature within 10 min from door closing.
Calculation	Recheck.

3. Readiness To Perform

- a. Familiarization.
 - i. Phase I: Standards—Not applicable.
 - ii. Phase II: Fortified samples—Random replicates of previously analyzed samples.
 - iii. Phase III: Check samples for analyst accreditation.
- b. Acceptability criteria.
See section J.1 above.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

4. Intralaboratory Check Samples

-
- a. System, minimum contents.
 - i. Frequency: 1 per week, not to exceed 20% of samples.
 - ii. Blind samples or random replicates chosen by supervisor after initial analysis.
 - iii. Records are to be maintained by analyst and reviewed by the supervisor and Laboratory QA Officer.
 - b. Acceptability criteria: refer to J.1 above.
 - If unacceptable values are obtained, then:
 - i. Stop all official analyses for that analyst.
 - ii. Investigate and identify probable cause.
 - iii. Take corrective action.
 - iv. Repeat Phase III of section J.3 if cause was analyst-related.
-

5. Interlaboratory Check Sample Program

- a. Laboratory responsibility: Analyze samples and report results promptly; maintain appropriate records.
 - b. Chemistry Division responsibility: Evaluate data and report promptly to participants.
 - c. Acceptability criteria. Refer to J.1 above.
-

6. Sample Acceptability and Stability

- a. Matrix: Meat, poultry, and processed products.
 - b. Sample receipt size, minimum: Size varies with sampling program. See Inspection Manual, Part 23.
 - c. Condition upon receipt: Cold and sealed from air.
 - d. Sample storage:
 - i. Time: 1 week.
 - ii. Condition: 4° C.
-

7. Sensitivity

- a. Lowest detectable level (LDL): NA
 - b. Lowest reliable quantitation (LRQ): 0.5%.
 - c. Minimum proficiency level (MPL): 0.5%.
-

AMINO ACID ANALYSIS OF MECHANICALLY SEPARATED SPECIES

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DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory

Nutritional value of any food product is derived from its content of protein, fat, and carbohydrate. The nutritional value of the contained proteins is based on the bioavailability and content of certain amino acids that are essential to nutrition. Current regulations list these essential amino acids as isoleucine, leucine, lysine, methionine, phenylalanine, threonine, and valine. Tryptophan is considered to be an essential amino acid by most nutritionists, but the current regulation does not list tryptophan because of the added cost of assay. However, if proper hydrolytic conditions are used, tryptophan content in a protein may be accurately determined with only a moderate increase in assay cost. Analysis of the nonessential amino acid cystine is not required by current regulations and is not included in this procedure.

To ensure maximum recovery of all amino acids, hydrolysis must be carried out under controlled conditions in the absence of oxygen. Thioglycolic acid, a reducing agent, is added to protect tryptophan and sulfur amino acids from oxidative destruction during hydrolysis. Norleucine acts as an internal standard to monitor sample handling losses.

Because mechanically separated (species) is made by crushing and grinding of marrow-containing bones, the nutritional value of the product can be diluted by the inclusion of excessive quantities of cartilaginous materials. Several amino acids that are present in collagen serve as indicators of collagen content. The most notable of these are hydroxylysine, hydroxyproline, proline, glycine, and alanine. Methionine content is often very low; cystine and tryptophan are usually absent.

The elution program developed for the amino acid analyzer separates all of the common amino acids plus hydroxyproline, hydroxylysine, norleucine, methionine sulfoxide and cysteic acid in a single run of 105 min. Traces of thioglycolic acid remaining in the hydrolysate do not interfere since thioglycolic acid elutes from the column well ahead of any amino acid.

Although tryptophan content in these products is very low, the Mark II computer system now in use on the Durrum D-500 analyzer gives adequate quantitation.

Interferences from fat and variations in moisture content of samples have been reduced by the preparation of acetone-chloroform powders of samples for analysis.

The instrument operator must be familiar with the operation and routine maintenance of the Durrum D-500 amino acid analyzer before beginning sample analysis. Each set of samples run should include random standards to check equipment function daily.

DETERMINATIVE METHOD

A. INTRODUCTION (Continued)

2. Applicability

This method can be used to determine the amino acid content of mechanically separated product made from meat or poultry.

Small variations in elution time of each peak (± 15 sec) routinely occur; therefore the names, elution times, and areas stored in computer memory on which quantitation of peaks is based should be the means from a ten-standard run. Also, because elution position and peak area are extremely pH-sensitive, each new batch of buffer or ninhydrin must be checked for its effect on standard values. A new set of peak areas and elution times must be run for each new batch of reagents.

In order to maintain quality assurance of assay, coefficients of variation of the means of the standard series placed in computer memory must be kept as low as possible, preferably no greater than three (3.0).

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

-
- a. Small table-top vise (clamp-on type) for mounting filling jig for sample holding units (SHU's).
 - b. Meat grinder, commercial model, Hobart or equivalent.
 - c. Plastic bags.
 - d. Balance, readable to 0.01 g, digital electronic type, 200-400 g capacity, Fisher #A-200 or equivalent.
 - e. Polypropylene test tubes, 25 mm id with fitted or screw tops (Falcon #2070), 50 mL capacity.
 - f. Tissuemizer, Tekmar Model SDT or equivalent.
 - g. 1 mL volumetric pipettes, Class A.
 - h. Vortex mixer or equivalent.
 - i. Disposable Pasteur pipettes, 9" length, and rubber bulbs.
 - j. Hydrolysis tubes, two-piece, with vacuum fitting (Kontes #K-896850), 18 mm x 140 mm, 25 mL capacity.
 - k. Heating block, multiple type, Lab-line or equivalent, 2 needed.
 - l. Thermometer, 0 to 150° C.
 - m. Vacuum pump, any make or model capable of at least 1.0 u final vacuum and capacity of at least 55 L/min free air.
 - n. McLeod gauge.
 - o. Heavy wall vacuum tubing 1/4" id.
 - p. Buchler Evapomix and adapters.
 - q. Infrared heat lamp.
 - r. Cold trap (Kontes #457500).
 - s. 18 x 150 mm Pyrex test tubes (Kimble #45048 or equivalent).
 - t. Test tube racks.
 - u. Wash bottle.
 - v. Dispensing pipette, Brinkman, 10 mL adjustable.
 - w. Polypropylene snap-top disposable test tubes, 17 x 100 mm (Curtin Matheson Scientific #252-981).

DETERMINATIVE METHOD

B. EQUIPMENT (Continued)

-
- x. Disposable microfilters, Gelman Acrodisc or Millipore; Gelman Acrodisc AN-450 with pre-filter, Gelman Instruments, Ann Arbor, MI or Millipore Millex SLHA 0250S, Millipore, Bedford, MA.
 - y. 10 mL syringes, plastic disposable with luer fitting.
 - z. pH meter, digital, readable to ± 0.01 pH unit.
 - aa. Labeling tape.
 - bb. Marker pens.
 - cc. Rubber tubing $\frac{1}{4}$ " id.
 - dd. Microliter syringe, 100 or 250 μ l Hamilton or Glenco.
 - ee. Parafilm.
 - ff. Heavy-duty ring stand.
 - gg. Analytical balance.
 - hh. Buchner funnel, 60 mL, with coarse-porosity fritted disk.
 - ii. Suction flask and vacuum adapters.
 - jj. Statistical calculator with capacity for calculation of standard deviation, coefficients of variance, linear regression, etc. (Texas Instruments SR51-II or equivalent)
-

2. Instrumentation

Durrum D-500 Amino Acid Analyzer and associated equipment, or equivalent.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and
Solution List

-
- a. Thioglycolic and mercaptoacetic acid—98 + % pure.
 - b. Thiodiglycol (2,2 thiodiethanol).
 - c. Hydrochloric acid, ACS reagent.
 - d. Na Citrate, ACS reagent.
 - e. Liquified phenol, reagent, MCB.
 - f. Pentachlorophenol preservative, Pierce.
 - g. Distilled water used in making buffers must be of high purity. Specific resistivity of at least four (4) megohms is recommended.
 - h. 0.2N Na Citrate buffer, pH 2.2 (sample and standard diluent).
 - i. 0.2N Na Citrate buffer, pH 3.00 ± 0.01 .
 - j. 0.2N Na Citrate buffer, pH 3.25 ± 0.01 .
 - k. 0.2N Na Citrate buffer, pH 4.25 ± 0.01 .
 - l. 1.1N Na Citrate buffer, pH 7.90 ± 0.01 .
 - m. Methyl cellusolve (ethylene glycol monomethyl ether) for use in cold trap.
 - n. Supply of dry ice. Dry ice used in cold trap during evacuation of samples prior to hydrolysis and for removal of HCl and water following hydrolysis.
 - o. Acetone, glass-distilled (Burdick & Jackson).
 - p. Chloroform, distilled-in-glass (Burdick & Jackson).
 - q. HCl, 6N (1 + 1).
 - r. Acetone:chloroform (3:1).
-

DETERMINATIVE METHOD

D. STANDARDS

Source

-
- a. Amino acid calibration standard (Pierce Chemical, Amino Acid Standard H, #20088).
 - b. Amino acids—amino acid kit from Pierce, Regis, or other supplier.
 - c. Amino acids—hydroxyproline, hydroxylysine, tryptophan, norleucine, methionine sulfoxide and cysteic acid—purchase separately if not in kit.
-

DETERMINATIVE METHOD

E. SAMPLE HANDLING

Sample Preparation

Grind samples for analysis at least 2 × by passage through a meat grinder fitted with a plate having 1/8" holes.

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE

Determination

-
- a. Weigh 2-3 g sample into 25 mm diameter tube.
 - b. Add 20 mL acetone:chloroform from dispensing pipette.
 - c. Homogenize 1 min with tissuemizer.
 - d. Pour blended sample into 60 mL Buchner funnel with a coarse-porosity frit.
 - e. Add second 20 mL portion of acetone:chloroform to sample tube and run blender again for a few seconds to clean blade. Add washings to Buchner funnel.
 - f. Apply vacuum and remove acetone. Continue vacuum until sample is air-dry.
 - g. When sample is dry (ca. 2-4 min), remove from funnel and place in labeled disposable polypropylene tube.
 - h. Weigh duplicate samples, 7.0 ± 0.1 mg (use disposable plastic weighing pan). Transfer weighed sample to labeled hydrolysis tube. Wash any adhering particles of sample from weighing pan into tube by dropwise addition of (1 + 1), HCl (0.5-1.0 mL).
 - i. Add 1.0 mL norleucine internal standard (2.5 μ moles/mL in pH 2.2 citrate buffer).
 - j. Add 100 μ l thioglycolic acid to hydrolysis tube.
 - k. Add 1.0 mL 12N (conc) HCl to each tube.
 - l. Assemble hydrolysis tubes, then fill cold trap with methyl cellulose dry ice mixture. Add dry ice in small pieces at first, waiting after each addition until violent frothing stops. Continue adding dry ice until trap is full. Do not turn on vacuum pump until trap is totally chilled. Check condensate reservoir and empty if any liquid is present. Rotate McLeod gauge so that mercury is in reservoir. Attach vacuum hose to first sample, open stopcock, and turn on vacuum pump.
 - m. Evacuate tube. Agitate or shake tube gently during evacuation to aid in release of dissolved oxygen. If bubbles begin to rise up walls of tube, shut off vacuum momentarily by closing stopcock of hydrolysis tube until bubbles break. Continue evacuating tubes until internal pressure drops to 50-100 u. Close stopcock and remove vacuum hose.
 - n. Attach hose to next sample tube, open stopcock, and evacuate as above. If pump oil is not contaminated and no leaks in tube parts or vacuum hose and connections are present, evacuation of each tube should require only three min.
 - o. After tubes are evacuated, place in heating block at $110 \pm 1^\circ$ C and hydrolyze for 24 hr.
 - p. After 24 hr, remove tubes from heating block and cool to room temperature.

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE (Continued)

-
- q. Open stopcock and release vacuum.
 - r. Use 9" disposable Pasteur pipette and transfer hydrolysate to clean labeled 18 x 150 mm pyrex tube.
 - s. Rinse hydrolysis tube with several small rinses of distilled water, adding each rinse to 18 x 150 mm tube.
 - t. Attach Evapomix adapter to top of tube and mount tube in Evapomix. Be sure cold trap is chilled before turning on vacuum. Flow cold water through Evapomix condenser. Adjust initial bath temperature of 40° C. Turn motor of Evapomix on and rotate tubes to spin liquid up walls of tube. Turn off vacuum pump; then open stopcock and begin evaporating liquid. Gradually increase bath temperature to 60° C. Keep cold trap filled with dry ice during evaporation to prevent vapors from entering vacuum pump and contaminating oil.
 - u. Evaporate samples to dryness, then remove each sample from Evapomix and wash down walls of tube with small amount of distilled water.
 - v. Remount Evapomix adapter on tube and again evaporate to dryness (or as near dryness as possible—thioglycolic acid does not evaporate readily).
 - w. When evaporation is complete, remove samples from Evapomix and add 5.0 mL pH 2.2 sample dilution buffer to each tube. Cover top of tube with square of Parafilm.
 - x. Mix sample well on vortex mixer. Be sure small reddish-brown button of thioglycollate disappears from bottom of tube.
 - y. Remove plunger from disposable syringe and attach disposable microfilter to end of barrel. Pour sample into syringe barrel, replace plunger, and filter sample into labeled snap-top disposable polypropylene tube.
 - z. Load 20 μ l of each diluted sample into cartridge (SHU) for analysis of common amino acids.
-

DETERMINATIVE METHOD

G. CALCULATIONS

Procedure

-
- a. Add up totals of essential amino acids and total amino acids.
 - b. Calculate essential amino acids, total amino acids ratio, i.e., essential amino acids = total of isoleucine, leucine, lysine, methionine, phenylalanine, threonine and valine. Divide total of above values (ng, mg, etc.) by total value (ng, mg, etc.) of isoleucine, leucine, lysine, methionine, phenylalanine, threonine, valine, alanine, arginine, aspartic acid, histidine, hydroxyproline, glutamic acid, glycine, proline, serine, and tyrosine, and multiply by 100. Do not include norleucine and other minor constituent amino acids in calculations. Express essential amino acid content as percent value.
 - c. All sample hydrolysates used to calculate essential amino acid content must have internal standard recovery of greater than 90%.
 - d. Average values of duplicate hydrolysates and report final answer as average of duplicate essential amino acid content measurements. Samples used for duplicate averaging must not differ from each other by more than 10%.
-

AMINO ACID ANALYSIS OF MECHANICALLY SEPARATED SPECIES/HPLC

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DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory

The nutritional value of any food product is derived from its content of protein, fat, and carbohydrate. The nutritional value of the contained proteins is based on the bioavailability and content of certain amino acids that are essential to nutrition. Current regulations list these essential amino acids as isoleucine, leucine, lysine, methionine, phenylalanine, threonine, and valine.

Tryptophan is considered to be an essential amino acid by most nutritionists, but the current regulation does not list tryptophan because of the added cost of assay charged for its analysis by commercial laboratories. However, if proper hydrolytic conditions are used, tryptophan content in a protein may be accurately determined with only a moderate increase in assay cost. Tryptophan analysis requires the modification of several steps within the methodology. These will be noted as they occur.

In addition, analysis of the nonessential amino acid cystine is not required by current regulations and is not included in this procedure.

In order to ensure as complete recovery as possible of all amino acids, hydrolysis must be carried out under controlled conditions in the absence of oxygen. Thioglycolic acid, a strong reducing agent, is added to protect tryptophan and sulfur amino acids from oxidative destruction during hydrolysis. Norleucine acts as an internal standard to monitor sample handling losses.

Because mechanically separated (species) is made by crushing and grinding of marrow-containing bones, the nutritional value of the product can be diluted by the inclusion of excess quantities of cartilaginous materials. Several amino acids that are present in collagen serve as indicators of collagen content. The most notable of these are hydroxylysine, hydroxyproline, proline, glycine, and alanine. Methionine content is often very low; cystine and tryptophan are usually absent.

The elution program developed for the high pressure liquid chromatographic separation of amino acids will also successfully separate hydroxyproline, proline, norleucine, methionine sulfoxide, and cysteic acid and all common amino acids in a single run of 90 min. Traces of thioglycolic acid remaining in the hydrolysate do not interfere since thioglycolic acid elutes from the column well before any of the amino acids. Although tryptophan content of these products is very low, adequate quantitation may be achieved by modification of the elution program. Interferences from fat and variations from moisture content have been obviated by the preparation of acetone-chloroform powders for analysis.

The instrument operator must be totally familiar with the operation and routine maintenance of the Waters Associates amino acid analyzer before beginning sample analysis.

The Waters HPLC Amino Acid Analysis System is an automated gradient liquid chromatograph tailored for the analysis of free amino acids. The amino acid separation is accomplished via the use of a combined pH-ionic strength gradient on a strong cation exchange column; the amino acids are derivatized with orthophthalaldehyde (OPA) following elution from the column and detected by fluorescence. The secondary amino acids proline and hydroxyproline are oxidized with hypochlorite prior to reaction with OPA.

2. Applicability

This method can be used to determine the amino acid content of mechanically separated product made from meat or poultry.

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

-
- a. Meat grinder, commercial model, Hobart or equivalent.
 - b. Plastic bags for sample storage.
 - c. Balance, Mettler, Model K7T, readable to ± 0.01 g, or equivalent.
 - d. Polypropylene test tubes, 25 mm id with fitted or screw tops (Falcon #2070), 50 mL capacity, or equivalent.
 - e. Tissuemizer, Tekmar Model SDT or equivalent.
 - f. 1 mL volumetric pipettes, Class A.
 - g. Vortex mixer, or equivalent.
 - h. Disposable Pasteur pipettes, 9" length, and rubber pipet bulbs.
 - i. Hydrolysis tubes, two-piece, with vacuum fitting—Kontes #K-896850, 18 mm \times 140 mm, 25 mL capacity.
 - j. Heating block, multiple type, Lab-line, or equivalent (need 2), with insert blocks having 18 mm holes.
 - k. Thermometer, 0 to 150° C.
 - l. Vacuum pump, direct drive, double stage—CSC Scientific 90703-001 or equivalent.
 - m. McLeod gauge.
 - n. Heavy wall vacuum tubing $\frac{1}{4}$ " id.
 - o. Buchler Evapomix and adapters.
 - p. Infrared heat lamp.
 - q. Cold trap (Kontes #457500).
 - r. 18 \times 150 mm Pyrex test tubes—Kimble #45048 or equivalent.
 - s. Test tube racks.
 - t. Wash bottle.
 - u. Dispensing pipette, Brinkman, 10 mL adjustable, or equivalent.
 - v. Polypropylene snap-top disposable test tubes, 17 \times 100 mm—Curtin Matheson #252-981 or equivalent.

DETERMINATIVE METHOD

B. EQUIPMENT (Continued)

-
- w. Disposable microfilters, Gelman Acrodisc or Millipore: Gelman Acrodisc AN-450 with pre-filter, Gelman Instruments, Ann Arbor, MI or Millipore Millex SLHA 0250S, Millipore, Bedford, MA.
 - x. 10 mL syringes, plastic disposable, with luer fitting.
 - y. pH meter, digital, Corning Model 125 or equivalent.
 - z. Labeling tape.
 - aa. Marker pens.
 - bb. Microliter, syringe, 100 μ l.
 - cc. Parafilm.
 - dd. Heavy-duty ring stand.
 - ee. Analytical balance, Mettler, Model H54AR, or equivalent.
 - ff. Buchner funnel, 60 mL, with coarse porosity fritted disk.
 - gg. Suction flask and vacuum adapters.
-

2. Instrumentation

Waters Associates Amino Acid Analysis System—Waters Associates, Milford, MA.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

1. Reagent and Solution List

-
- a. Thioglycolic acid (Mercaptoacetic acid) 98+ % pure.
 - b. o-phthalaldehyde (OPA)—Alfa Products (16344), or equivalent.
 - c. Hydrochloric acid—Baker Ultrex grade.
 - d. Sodium citrate, ACS reagent grade—Baker 1-3646, or equivalent.
 - e. Brij 35-30% solution—Fisher CS-285-3, or equivalent.
 - f. Distilled water used in making buffers must be of high purity—minimum specific resistivity of at least ten megohms is required.
 - g. Potassium hydroxide ACS—Baker 3140.
 - h. Sodium chloride ACS reagent—Fisher S-671, or equivalent.
 - i. Sodium hydroxide ACS reagent—Fisher S-318, or equivalent.
 - j. Methyl cellosolve (ethylene glycol monomethyl ether), for cold trap.
 - k. Dry ice for cold trap.
 - l. Acetone, distilled in glass—Burdick and Jackson, or equivalent.
 - m. Chloroform, distilled in glass—Burdick and Jackson, or equivalent.
 - n. Acetone: Chloroform (3:1).
 - o. Boric acid granular—Baker (00945).
 - p. 3-mercaptopropionic acid—Sigma (M 6750), or equivalent.
 - q. Methanol—Burdick and Jackson, or equivalent.
 - r. 2-propanol—Burdick and Jackson, or equivalent.
 - s. Sodium hypochlorite solution, 5%—Aldrich Chemicals 23,930-5, or equivalent.
-

2. Composition of Elution Buffers and Post Column Solutions

NOTE: All formulations are for 1-L quantities.

- a. Buffer A: 0.2N Na⁺, 2% 2-propanol, pH 2.95.
 - i. Sodium citrate dihydrate—19.6 g.
 - ii. 2-propanol—20.0 g.
 - iii. Hydrochloric acid (Ultrex) to pH 2.95.

DETERMINATIVE METHOD

C. REAGENT AND SOLUTIONS (Continued)

-
- b. Buffer B: 1.1N Na⁺, pH 7.1-7.4.
 - i. Sodium citrate dihydrate—19.6 g.
 - ii. Sodium chloride—52.5 g.
 - iii. Hydrochloric acid (Ultrex) to pH 7.1-7.4.
 - c. Post column reaction buffer: 0.5M boric acid—KOH, pH 10.2-10.7.
 - i. Boric acid—30.5 g.
 - ii. Potassium hydroxide—26.3 g.
 - d. OPA solution.
 - i. Orthophthaldehyde—700 mg.
 - ii. Methanol—10 mL.
 - iii. 3-mercaptopropionic acid—2.0 mL.
 - iv. BRIJ 35—1.0 mL.
 - v. 0.5 M boric acid—KOH buffer—1.0 L.

NOTE: Dissolve the OPA and 3-mercaptopropionic acid in the methanol and then combine with boric acid-KOH BRIJ 35 solution.

- e. Hypochlorite solution.
 - i. Sodium hypochlorite—2.0 mL.
 - ii. BRIJ 35—1.0 mL.
 - iii. 0.5M boric acid —KOH buffer—1.0 L.

NOTE: Degas K borate-BRIJ 35 mixture thoroughly before adding sodium hypochlorite.

3. Preparation of Buffers and Solutions

-
- a. Dissolve salts in purified water at 90% of final volume.
 - b. Adjust pH with hydrochloric acid.
 - c. Make up to final volume in volumetric flask using purified water.
 - d. Filter solution through 0.45 μ aqueous compatible filter.

DETERMINATIVE METHOD

C. REAGENT AND SOLUTIONS (Continued)

-
- e. Transfer eluent to amber bottles and purge for 15 min with nitrogen.
 - i. Buffer A and B may be made up in 4 L quantities and stored two weeks in the refrigerator.
 - ii. Post column reaction buffer may be prepared in 4 L quantities and is stable at room temperature.
 - iii. The OPA and hypochlorite solutions must be stored in an inert atmosphere and prepared fresh every 48 hr.
-

DETERMINATIVE METHOD

D. STANDARDS

1. Source

-
- a. Amino acid calibration standard (Pierce Chemical, Amino Acid Standard H, #20088).
 - b. Amino acids—purchase amino acid kit from Pierce, Regis, or other supplier.
 - c. Amino acids—hydroxyproline, hydroxylysine, tryptophan, norleucine, methionine sulfoxide and cysteic acid—purchase separately if not in kit.
-

2. Preparation of Standard

-
- a. Amino acid standard is prepared by mixing 1 mL of calibration standard H, 1 mL of 2.5 μ M/mL hydroxyproline and 1 mL of 2.5 μ M/mL norleucine. The mixture is made up to a final volume of 10 mL using buffer A. Store the standard in the refrigerator and prepare fresh every 2 weeks. Note that with this dilution and a 20 μ L injection the column load is 5.0 nanomoles per amino acid.
 - b. For addition of hydroxyproline and norleucine to the mixed amino acid standard, dissolve each in 0.1N HCl solution at 2.5 μ M/mL (store in refrigerator). Other amino acids listed under Section D. 1. c may be added to the mixed amino acid standard for peak identification purposes only. If tryptophan is to be added, it must be dissolved in a neutral or basic solution such as the "B" buffer, since it is degraded in acidic solution. A special elution program is required for tryptophan quantitation.
-

NOTE: Tryptophan analysis:

- a. Amino acid standard is prepared by pipetting 1 mL of calibration standard H, 1 mL of 2.5 μ M/mL hydroxyproline, 1 mL of 2.5 μ M/mL norleucine and 1 mL of 0.625 μ M/mL tryptophan into a 10 mL volumetric flask. Make up to final volume using buffer "A." Store the standard in the refrigerator. Make fresh weekly. Note that with this dilution and a 20 μ L injection, the column load is 1.25 nanomoles for tryptophan and 5.0 nanomoles for all other amino acids.
 - b. For addition of tryptophan to the mixed amino acid standard dissolve tryptophan in buffer "B" at 0.625 μ M/mL. Store in refrigerator. Make fresh monthly.
-

MSS2
May, 1993

DETERMINATIVE METHOD

E. SAMPLE HANDLING

Sample Preparation

Grind samples for analysis at least 2 × by passage through a meat grinder fitted with a plate having 5/64" holes. (For atypical samples containing large amounts of skin or bone, blend in a commercial blender, after pre-chilling both blender and samples with liquid nitrogen.)

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE

1. Determination

- a. Weigh 2-3 g sample into 25 mm diameter tube.
- b. Add 20 mL acetone:chloroform from dispensing pipette.
- c. Homogenize 1 min with tissuemizer.
- d. Pour blended sample into 60 mL Buchner funnel.
- e. Add second 20 mL portion of acetone:chloroform to sample tube and blend for a few seconds to clean blade. Add washings to Buchner funnel.
- f. Apply vacuum and filter off acetone chloroform solvent. Continue vacuum until sample is air-dry. If after drying sample has sticky texture, blend again with solvent and air-dry. A dry, free-flowing powder must be produced.
- g. When sample is dry (ca. 2-4 min) transfer to a clean, labeled disposable polypropylene tube.
- h. Weigh duplicate samples, 10.0 ± 0.1 mg (use disposable plastic weighing pan). Transfer weighed sample to labeled hydrolysis tube. Wash any adhering particles of sample from weighing pan into tube by dropwise addition of (1 + 1) HCl (0.5-1.0 mL).
- i. Add 1.0 mL norleucine internal standard ($2.5 \mu\text{M}/\text{mL}$ in 0.1N HCl).
- j. Add 150 μL thioglycolic acid to hydrolysis tube.
- k. Add 1.5 mL 12N (conc) HCl to each tube, followed by 0.5 mL deionized water.
- l. Assemble hydrolysis tubes, then fill cold trap with methyl cellusolve and dry ice mixture. Add dry ice in small pieces at first, waiting after each addition until violent frothing stops. Continue adding dry ice until trap is full. Do not turn on vacuum pump until trap is totally chilled. Check condensate reservoir and empty if any liquid is present. Rotate McLeod gauge so that mercury is in reservoir. Attach vacuum hose to first sample, open stopcock, and turn on vacuum pump.
- m. Immerse tubes in ice bath for first 2-3 min of evacuation period. Agitate or shake tube gently during evacuation to aid in release of dissolved oxygen. If bubbles begin to rise up walls of tube, shut off vacuum momentarily by closing stopcock of hydrolysis tube until bubbles break. Continue evacuating tubes until internal pressure drops to 50-100 μ . Close stopcock and remove vacuum hose.
- n. Attach hose to next sample tube, open stopcock, and evacuate as above. If pump oil is not contaminated and no leaks in tube parts or vacuum hose and connections are present, evacuation of each tube should be complete in 2-4 min.
- o. After tubes are evacuated, place in heating block at 145° C and hydrolyze for 4 hr. Tubes should be protected from all drafts during hydrolysis period to prevent cooling of upper part of tube. A convenient cover can be fashioned by covering a wire mesh test tube basket with aluminum foil. Temperature in block must be maintained at a minimum of 145° C, but must not exceed 150° C during hydrolysis period.

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE (Continued)

-
- p. After 4 hr, remove tubes from heating block, and cool to room temperature.
 - q. Open stopcock and release vacuum.
 - r. Use 9" Pasteur pipette and transfer hydrolysate to clean labeled 18 x 150 mm pyrex tube.
 - s. Rinse hydrolysis tube with small rinses of distilled water, adding each rinse to 18 x 150 mm tube. Restrict volume used for rinsing to no more than 1.0 mL.
 - t. Attach Evapomix adapter to top of tube and mount tube in Evapomix. Be sure cold trap is chilled before turning on vacuum. Flow cold water through Evapomix condenser. Adjust initial bath temperature to 60° C. Turn motor of Evapomix on and rotate tubes to spin liquid up walls of tube. Turn on vacuum pump; then open stopcock gradually to begin evaporating liquid. Total volume of hydrolysate plus rinses in evaporator tube should not exceed 5 mL to prevent sample losses from bumping during early stages of evaporation. Keep cold trap filled with dry ice during evaporation to prevent vapors from entering vacuum system and contaminating pump oil. Turn on infrared heat lamp to assist in driving vapor out of vacuum adapter bulbs.
 - u. Evaporate samples to near dryness; then remove each sample tube from Evapomix and wash down walls of tube with small amount of distilled water.
 - v. Remount Evapomix adapter on tube and again evaporate to dryness. NOTE: thioglycolic acid does not evaporate completely.) Sharp acrid odor of HCl should be absent from hydrolysates at end of evaporation.
 - w. When evaporation is complete, remove samples from Evapomix and add 10.0 mL of "A" buffer to each tube. Cover top of tube with square of Parafilm.
 - x. Mix sample well on vortex mixer. Be sure reddish-brown button of thioglycolate disappears from bottom of tube.
 - y. Remove plunger from disposable syringe and attach disposable microfilter to end of barrel. Pour sample into syringe barrel, replace plunger, and filter sample into labeled snap-top disposable polypropylene tube. Store filtered hydrolysates in refrigerator. Stable one week. Dried undiluted hydrolysates may be stored in a freezer for an indefinite period.
-

2. Daily HPLC Equipment Start-Up

- a. Turn switches to the following indicated positions:
 - i. Solvent delivery system, pumps A and B: switch toggle switch "ON" with flow control set at 0.0 mL/min on digital meter.
 - ii. Post column reaction pumps: switch toggle switch on each pump ON and set unit switch to auto.
 - iii. Temperature control module: press power button ON and set temperature to 60° C. Adjust control as needed.
 - iv. WISP: Press power button ON.

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE (Continued)

-
- v. Data module: Press power button ON.
 - vi. System controller: Press power button ON.
 - vii. M-420 Fluorometer: Press power button ON. Set attenuator at 2. Turn SPAN completely clockwise.
- b. Program loading.
- i. Insert tape into system controller.
 - ii. After the Waters' logo appears, press NEXT MOD to access the system monitor page.
 - iii. Type date as a 6 digit parameter, press NEXT VAL, type time as a 6 digit parameter, press NEXT VAL, type an operator code and press ENTER.
 - iv. Press NEXT PAGE 4 times to access the tape operation page.
 - v. Type "load," press ENTER.
 - vi. Type "AAA.200," press ENTER. Program loading is complete when "to continue press enter" appears on the screen. At this point all system set points have been automatically entered by the tape. See Section F.5 for a detailed listing of parameters.

NOTE: *Tryptophan analysis*: Type "AAA.006," press ENTER.

- c. Pump priming and purging.
- i. Place inlet lines in appropriate buffer bottles positioned at a level above the pumps.
 - ii. Press ENTER, press NEXT MOD to access the pump monitor page.
 - iii. Press NEXT PAGE to access initial condition page.
 - iv. Open relief valve on pump "A."
 - v. STOP! Is the relief valve open? Type a flow of 10 mL/min, 100% A, and press ENTER. Prime the M6000 A with buffer A using a 10 mL syringe on the draw-off valve. A steady stream of effluent should be seen from the relief valve tube.
 - vi. Type 0% A, 100% B, and press ENTER. Prime the B pump with buffer B.
 - vii. Type a flow of 0 mL/min, 100% A, 0% B and press ENTER.
 - viii. Press NEXT PAGE until pump monitor page is accessed.
 - ix. STOP! Is the flow 0? If no flow is present, close relief valve on pump A.

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE (Continued)

- x. Prime the post column reaction pumps with appropriate solutions by drawing 20 mL of solution through the pump using a syringe attached to the draw-off valve.
- d. Column and system equilibration.
 - i. After the column has reached 60° C, access the pump monitor page and press ENTER.
 - ii. Type 2 for editing pump set, type 2 for running pump set, and press ENTER. The system is now operating at a flow of 0.5 mL/min of 100% buffer A. The pressure should stabilize at 700 to 1200 psi.
 - iii. Allow system to equilibrate for 30 min.

3. Calibration Table and Standard Plan

- a. Place calibration standard in position 1 of WISP.
- b. Press RUN/STOP button on WISP and allow 90 min for standard run.
- c. Using the above standard run prepare a calibration table as illustrated below.

Table I
Calibration Worksheet

Amino Acid	ID Numbers	Retention Time	Amount ng injected
Hydroxyproline	1002	Enter values	655
Aspartic acid	1002	"	665
Threonine	1001	"	595
Serine	1002	"	525
Glutamic Acid	1002	"	735
Proline	1002	"	575
Glycine	1002	"	375
Alanine	1002	"	446
Valine	1001	"	585
Methionine	1001	"	745
Isoleucine	1001	"	655
Leucine	1001	"	655
Norleucine	1003	"	100†
Tyrosine	1002	"	905
Phenylalanine	1001	"	825
Lysine	1001	"	730
Histidine	1002	"	775
Arginine	1002	"	870
Tryptophan‡	1001	"	255

† The actual injected quantity is 655 ng; 100 is entered for this value to allow the report to contain the percent recovery for norleucine.

‡ Normal verification analyses do not require analysis of tryptophan.

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE (Continued)

-
- d. Enter the values of the calibration table into the data module.
 - i. Press CLEAR, ENTER.
 - ii. Press 10, ENTER.
 - iii. Press 1, ENTER. This erases any previous calibration tables.
 - iv. Press NEXT (ID). Enter ID number of first amino acid from table.
 - v. Press NEXT (RT). Enter retention time of first amino acid.
 - vi. Press NEXT (CO). Enter concentration of first amino acid.
 - vii. Press NEXT, NEXT (ID). Enter ID number of second amino acid. Continue until table is complete.
 - viii. When last amino acid has been entered press ENTER three times.
 - ix. List and check calibration table by entering 99 ENTER, 3 ENTER.
 - x. A wrong value may be removed by reentering the entire line as is, except enter 0 for concentration. This will remove the entire line. A line cannot be overwritten for correction. A new line may be added at any time.
 - e. Enter the recalibration sequence into module. The recommended sequence is to average two standards every 14 samples.
 - i. Enter a value of 0 for parameter 34.
 - ii. Enter a value of 1 for parameter 36.
 - iii. Enter a value of 2 for parameter 37.
 - iv. Enter a value of 14 for parameter 38.
 - v. Enter a value of 0 for parameter 39.
 - vi. Load the WISP tray such that standards are in position 1 and 15.
 - vii. Calibration sequence can be reinitiated by entering the value for parameter 37.

NOTE: *Tryptophan analysis:*

- e. Enter the recalibration sequence into module. The recommended sequence is to average two standards every 14 samples.
 - i. Enter a value of 1001 for parameter 33.
 - ii. Enter a value of 0 for parameter 34.
 - iii. Enter a value of 2 for parameter 36.

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE (Continued)

-
- iv. Enter a value of 2 for parameter 37.
 - v. Enter a value of 14 for parameter 38.
 - vi. Enter a value of 0 for parameter 39.
 - vii. Enter a value of 68 for parameter 73.
 - viii. Load the WISP tray such that standards are in positions 1 and 15.
 - ix. Calibration sequence can be reinitiated by entering the value of parameter 37.

NOTE: Parameter 38 is the number of samples before re-calibration. If more frequent re-calibration is used, the values in v. and viii. must be changed accordingly.

NOTE: Parameter 73 is one-half noise rejection. The 68.00 time cited in the note in Section F.5. is the time of return to baseline following histidine elution for the particular column in use at CDLB. This time must be determined by the analyst for each column and the value in min entered in the external events table in F.5. The reduction to one-half of the noise rejection parameter facilitates better quantitation of the small quantities of tryptophan present in MS(S) samples.

**4. Analysis and
Calculations**

-
- a. With samples and standards loaded in WISP, initiate the analysis by pushing the RUN/STOP button on the WISP.
 - b. The total weight of essential amino acids will be reported as Group 1, nonessentials as Group 2, and percent norleucine in Group 3 at the bottom of each report.
 - c. Weight percent essential amino acids is calculated by dividing Group 1 value by the sum of Group 1 and Group 2 value.
 - d. Norleucine recovery value must be greater than 90%.
 - e. Report percent essential amino acids as average of duplicate assays. Duplicates may be averaged only if percent amino acids in samples differs by less than 10%.
-

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE (Continued)

5. System Controller and WISP Parameters

Pump Controller Conditions

Standby Conditions for run set 02

Pressure: 0000 Delay Time: 0
High Limit: 2500 Plot: %B

Pump	Solvent	Comp
A	Buf A	2.95
B	1.1 Na	7.10
C		0

Initial Conditions/Gradient Table

Time	Flow	%A	%B	%C	Curve
Initial	.50	100	0	0	*
45.00	.50	20	80	0	08
60.00	.50	0	100	0	08
80.00	.50	100	0	0	11
120.00	.20	50	50	0	11

External Events

No.	Description
1	Hypo
2	OPA

Time	No.	Status
10.00	1	On
10.10	2	On
85.00	1	Off
85.10	2	Off

Sample POS 00. Inj volume 0020, No of Inj 1 *, Run Time 00.00, AM WISP codes generated.

* Note: Standard positions 1 and 15 are set at 2 injections/standard.

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE (Continued)

NOTE: *Tryptophan analysis*: Change high pressure limit to 1500. Modify external events table as follows:

Time	Number	Status
0.10	1	On
0.20	2	On
68.00	1	Off
110.00	2	Off

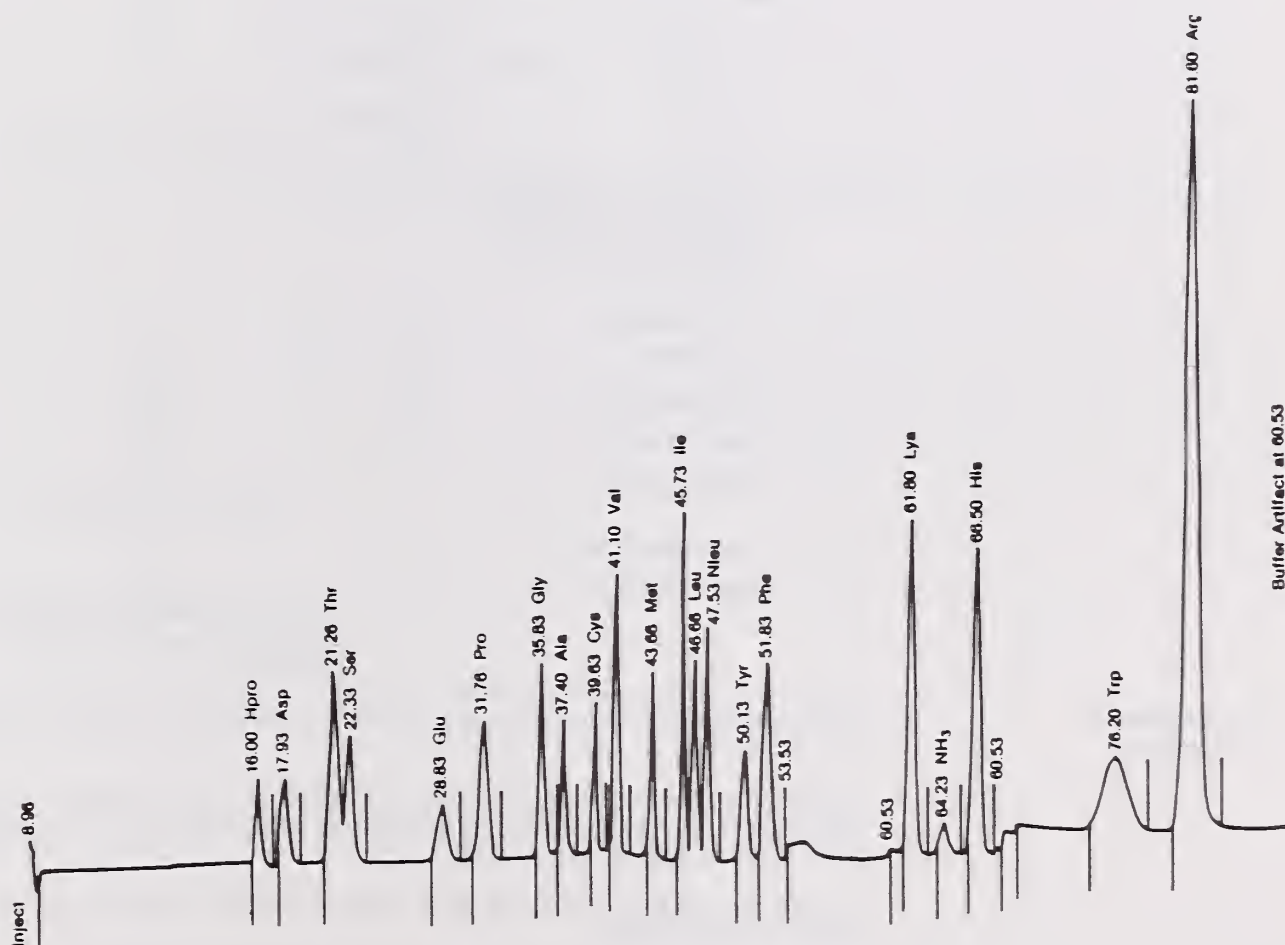
Note that with this modification both tryptophan and arginine are quantitated with hypochlorite oxidizer off. The relative quantity of tryptophan in the standard is reduced for closer quantitation of the small amounts of tryptophan present in these sample types. Arginine appears as a very large peak because of absence of oxidizer, but is adequately quantitated. See enclosed standard chromatogram on the following page.

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE (Continued)

Column Load: Trp 1.25 nanomoles
All other amino acids
5.0 nanomoles

Fluorometer: 4X, Span 40%



DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE (Continued)

6. Data Module Parameters	Code No.	Description	Symbol	Value
	0	Date	dA	Auto set via system controller
	1	Time of Day	ti	Auto set via system controller
	2	Chart Speed	CS	0.25
	3	Plot Mode	PL	0
	4	Pen 2	P2	0
	5	Pen 1 Zero	O1	10
	6	Pen 2 Zero	O2	10
	7	Auto Zero	Ao	1
	8	LC/GPC	LC	1
	9	Calib/Anal	CA	(Set via parameter 37)
	10-14	Calibration parameters discussed in Calibration Table and Stand Run Section F.3.		
	20	Auto Parameter	AP	1
	21	Peak Width	P—	20
	22	Noise Rejection	Nr	250
	23	Area Rejection	Ar	1000
	33	Report Format	—	100 (for a short report 0)
	45	Run number start	—	0
	63	Report Format	—	1000 (this configuration is required to prepare types for short report 0).
<hr/>				
7. Instrument Shutdown	a.	On a weekly basis, flush the column with 0.1N NaOH for 30 min at a flow of 0.5 mL/min.		
	b.	During short periods of inactivity, the equipment is programmed to reduce flow of buffer A and buffer B to a total flow of 0.2 mL/min. Post column pumps will automatically turn off.		
	c.	For 2-4 day shutdown periods, flush column with 0.2N NaOH and turn off power to all modules.		
	d.	For periods longer than one week, refer to the operator's manual.		

LACTOSE

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DETERMINATIVE METHOD

A. INTRODUCTION

Theory

Nonfat dry milk (NFDM) or calcium-reduced dry skim milk (CRDSM), each consisting of approximately 50% lactose, is added to meat food products as a binder. In this method, the amount of added NFDM or CRDSM is determined by analyzing the product for its lactose content.

A thorough understanding of the basic chemical principles underlying this determination helps lessen the difficulties of the analysis. We use washed baker's yeast (*Saccharomyces cerevisiae*) to ferment all reducing sugars other than lactose and maltose. If corn syrup or corn syrup solids have been added to the product in addition to NFDM or CRDSM, a maltose-acclimated yeast must be prepared to ferment the maltose. If active dry yeast is used in lieu of baker's yeast, it is important to add the yeast to the water when washing the yeast. The reverse procedure (adding water to the yeast) will destroy approximately 50% of the yeast population. Yeast consists of living, unicellular plant organisms and the dry variety must be thoroughly wet prior to stirring. It is necessary to test the yeast to be sure of its viability or potency. The acclimation procedure requires care in keeping the temperature from exceeding 30° C during incubation. The motor of a mechanical stirrer, for example, could add heat to the incubation oven.

This procedure is based upon the analysis of a labile constituent of NFDM or CRDSM. Lactose can be readily oxidized to lactic acid by certain microorganisms, especially *Streptococcus lactis*, which is present in muscle tissue. Lactose can also be quickly hydrolyzed to glucose and galactose by the action of hot, dilute acids. These reactions will, if they occur, cause analytical results to be low, since NFDM or CRDSM is calculated on the basis of the amount of lactose found. This analysis should be initiated as soon as the sample is ground and, if possible, completed on the same day.

DETERMINATIVE METHOD

B. EQUIPMENT

Apparatus

All apparatus may be substituted with an equivalent.

- a. Centrifuge—Model C-6000 (IEC 3504).
 - b. Swinging Bucket Rotor—IEC 256
 - c. Central adapter for 250 mL tubes—IEC 5780.
 - d. Centrifuge tubes—250 mL conical bottom with cap Fisher #05-538-53.
 - e. Cushion for 05-538-53 tubes—Fisher #05-538-53A.
 - f. Incubator—6000 series standard lab incubator—Fisher #11-683-655D.
 - g. Precision controlled rheostat heater—Fisher #11-425.
 - h. Standard laboratory glassware.
-

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

**Reagent and
Solution List**

- a. Washed yeast suspension.
 - i. Mix four cakes of baker's yeast (or 30 g of active dry yeast) to a smooth suspension with 300 mL of distilled H_2O (if active dry yeast is used, the yeast must be added to the water).
 - ii. Centrifuge for 5 min and discard aqueous layer. Repeat four more times, or until supernatant is clear following centrifugation.
 - iii. Suspend by stirring the yeast in distilled H_2O , dilute to 200 mL with distilled H_2O , and refrigerate at about 4° C.
- b. Acclimated yeast suspension.
 - i. Prepare acclimating medium by dissolving each of the following ingredients in a small amount of distilled H_2O and adding, in the order given, to 1,000 mL of distilled H_2O .
 - (a) 2.0 g anhydrous $MgSO_3$.
 - (b) 4.0 g NH_4Cl .
 - (c) 2.0 g anhydrous K_2HPO_4 .
 - (d) 1.0 g KCl .
 - (e) 0.04 g $FeSO_4 \cdot 7H_2O$.
 - (f) 1.4 g peptone.
 - (g) 40.0 g technical maltose.
 - ii. Dilute to 2 L, warm, and filter. Bring filtrate to a rolling boil and cool to room temperature.
 - iii. Shake well the washed yeast suspension obtained in a; remove 100 mL and centrifuge.
 - iv. Discard the aqueous layer, add the washed yeast to 1 L of the acclimating medium, and incubate for approximately 24 hr at 30° C, stirring frequently the first few hours.
 - v. Separate yeast by decanting and centrifuging. Wash twice with distilled H_2O and repeat incubation with the remaining 1 L of acclimating medium.
 - vi. Separate yeast again, wash 4 or 5 times with distilled H_2O , suspend yeast in distilled H_2O , dilute to 100 mL with distilled H_2O , and refrigerate at about 4° C.

NOTE: To determine the viability and potency of the acclimated yeast suspension: Weigh 500 mg of maltose and 800 mg of dextrose and transfer to a 100 mL volumetric flask. Dilute to volume with distilled H_2O , stopper, and mix well. Pipet a 10 mL aliquot into a 50 mL volumetric flask and

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS (Continued)

proceed through the incubation procedure. Boiling 10 mL of this solution (following centrifugation) with 20 mL of Benedict's solution for exactly 3 min should yield no precipitate or suspension of Cu_2O , indicating that the yeast fermented the sugars. If a precipitate or suspension occurs, the yeast should be discarded. This is the easiest procedure for determining that the yeast is "working" properly. [If testing washed yeast, weigh only 800 mg of dextrose. Do not use any maltose.]

- c. Dilute hydrochloric acid (1 + 4): One volume conc. HCl + 4 volumes distilled H_2O .
 - d. Phosphotungstic acid: 20% w/v.
 - e. Chlorophenol red indicator: Dissolve 0.1 g chlorophenol red in 2.4 mL of 0.1N NaOH and dilute to 250 mL with distilled H_2O .
 - f. Bromthymol blue indicator: Dissolve 0.1 g bromthymol blue in 1.6 mL of 0.1N NaOH and dilute to 250 mL with distilled H_2O .
 - g. Buffer solution, pH 4.8.
 - i. Prepare 0.1M citric acid (19.21 g/L) and 0.2M Na_2HPO_4 (28.4 g anhydrous/L).
 - ii. Mix solutions in proportions of 10.14 mL citric acid to 9.86 mL Na_2HPO_4 and adjust to pH 4.8, using a pH meter.
 - iii. Store in refrigerator and discard if solution becomes turbid.
 - h. Benedict solution.
 - i. Dissolve 16 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 150 mL of distilled H_2O .
 - ii. Dissolve 150 g sodium citrate dihydrate, 130 g anhydrous Na_2CO_3 , and 10 g NaHCO_3 in 650 mL of distilled H_2O .
 - iii. Combine the two solutions, cool, dilute to 1 L with distilled H_2O , and filter.
 - i. Dilute acetic acid: Dilute 240 mL glacial acetic acid to 1 L with distilled H_2O .
 - j. Dilute phosphoric acid: Dilute 240 mL phosphoric acid to 1 L with distilled H_2O .
 - k. Iodine standard solution: Dissolve 10.2 g KI in minimum quantity of distilled H_2O and use this solution as a solvent for 5.08 g I_2 . Filter, if necessary, through a glass fiber filter paper and dilute to 1 L with distilled H_2O .
 - l. Sodium thiosulfate standard solution: Dissolve 9.92 g $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ in recently boiled, cooled distilled H_2O and 0.1 g Na_2CO_3 , and dilute to 1 L with distilled H_2O .
 - m. Starch indicator solution: Triturate 2 g of soluble starch and 10 mg HgI_2 with a small amount of distilled H_2O . Add the suspension slowly to 500 mL boiling distilled H_2O and boil until clear.
-

DETERMINATIVE METHOD

D. STANDARDS

Preparation of Standards

-
- a. Lactose standard solution: Dissolve 1.5789 g lactose monohydrate in distilled H₂O and dilute to 1 L with distilled H₂O (10 mL = 15 mg anhydrous lactose).
 - b. Dextrose standard solution: Dissolve 1.500 g dextrose in distilled H₂O and dilute to 1 L with distilled H₂O (10 mL = 15 mg dextrose).
-

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE

1. Determination

- a. Weigh 20.0 sample into a 200 mL volumetric sugar flask.
- b. Add 50 mL distilled H₂O, stir or shake to break up any lumps, and heat on steam bath for 30 min.

NOTE: The small amount of water added to the 20 g sample should be at room temperature when added to the sugar flask. If it is not, coagulation of the meat and milk protein might occur, making it difficult to macerate the sample and leach out the lactose.

- c. Cool to room temperature, add 20 mL dilute HCl, and dilute to volume, using bottom of fat layer as meniscus.

NOTE: If following the 30-min heating time on the steam bath the flask and contents are not cooled to room temperature prior to adding the HCl, loss of lactose may take place by hydrolysis.

- d. Add 10 mL of 20% phosphotungstic acid solution, mix, let stand for a few minutes, and filter through a moistened filter paper.
- e. Pipet 40 mL filtrate into a 50 mL volumetric flask.
- f. If corn syrup or corn syrup solids are absent, neutralize just to the acid side of bromthymol blue indicator, dilute to volume with distilled H₂O, and mix.
- g. If corn syrup or corn syrup solids are present, neutralize just to the acid side of chlorophenol red indicator, add 5 mL of the buffer solution, dilute to volume with distilled H₂O, and mix.
- h. Transfer about 40 mL of this solution to a centrifuge tube to which 5 mL of yeast suspension (washed yeast if corn syrup or corn syrup solids are absent; acclimated yeast if either one is present) has been added and from which the H₂O has been separated.
- i. Mix yeast and sample well, and incubate washed yeast for 1 hr at 30° C or acclimated yeast for 3 hr at 30° C, stirring frequently.
- j. Centrifuge.
- k. Pipet 10 mL of clear supernatant into a 300 mL Erlenmeyer flask, add 20 mL of Benedict solution, cover with watch glass and bring to boil in 3-5 min, and boil for exactly 3 min.
- l. Remove from heat, cool rapidly, and add 100 mL distilled H₂O and 10 mL dilute acetic acid slowly while swirling. Keep covered with watch glass.

NOTE: The reducing portion of this method is extremely critical because it involves an empirical procedure. The 3-min boiling time must be strictly adhered to, and the flask should be cooled rapidly following boiling; for example, invert a beaker over the neck of the flask and allow a stream of cold tap water to flow over the flask.

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE (Continued)

-
- m. Add a definite volume of standard iodine solution (15 mL for about 1.5% lactose, or 30% excess) and agitate to dissolve the Cu_2O . Keep covered with watch glass.
 - n. Allow flask to stand at least 5 min. With watch glass just ajar, add 20 mL of dilute phosphoric acid solution. Slowly swirl to mix. Keep watch glass on. Rinse underside of watch glass into flask with distilled water before titrating excess iodine with standard sodium thiosulfate solution, using starch as an indicator.

NOTE: The titration should be performed immediately after the addition of the H_3PO_4 to avoid any possible loss of I_2 . The use of more costly iodine flasks will also serve to avoid loss of I_2 .

- o. Determine $\text{I}_2:\text{Na}_2\text{S}_2\text{O}_3$ ratio by using 10 mL of distilled water and carry through determination as above, beginning with step k "...add 20 mL of Benedict solution..."

$$\text{I}_2:\text{Na}_2\text{S}_2\text{O}_3 \text{ ratio} = \frac{\text{Volume I}_2 \text{ (mL)}}{\text{mL Na}_2\text{S}_2\text{O}_3} = A$$

- p. Determine lactose: I_2 ratio by using 10 mL of standard lactose solution and carrying through determination as above, beginning with step k "...add 20 mL of Benedict solution..."

$$\text{Lactose}:\text{I}_2 \text{ ratio} = \frac{15 \text{ mg lactose}}{\text{mL I}_2 - (\text{mL Na}_2\text{S}_2\text{O}_3)(A)} = B$$

2. Dextrose Determination

This procedure may also be used to determine total reducing substances (total sugars calculated as dextrose) in a sample by performing the method as above, except for the following changes.

- a. Add 20 mL dilute HCl at step 1.b rather than 1.c.
 - b. In step 1.e, dilute filtrate to 50 mL.
 - c. Skip steps 1.f through 1.j and proceed as in 1.k.
 - d. Determine the dextrose: I_2 ratio in step 1.p by using the dextrose standard (section D, item b) instead of the lactose standard.
-

DETERMINATIVE METHOD

G. CALCULATIONS

1. Procedure

$$\% \text{ Lactose} = \frac{100(\text{mL of } I_2 \text{ added to flask} - (A)(\text{mL of } Na_2S_2O_3 \text{ required for back titration}))(B)}{C}$$

A & B = Ratios defined above.

C = mg of sample in aliquot (consider the volume of the original sample solution as 200 mL rather than 210 mL to take into account the volume occupied by the sample).

% NFDM or % CRDSM = (% lactose \times 2) - Correction

Correction: 0.4% in the absence of corn syrup or corn syrup solids, and 0.8% in the presence of corn syrup or corn syrup solids.

NOTE: If section F.2 is performed, total reducing sugars calculated as dextrose may be calculated in the same manner, substituting the dextrose: I_2 ratio for B in the calculation.

2. Reference

Official Methods of Analysis of the Association of Official Analytical Chemists, 15th Edition, 927.07.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Lactose Determination		
2. Protective Equipment	Safety glasses, plastic gloves, and lab coat.		
3. Procedure Steps		<u>Hazards</u>	<u>Recommended Safe Procedures</u>
	C. Reagents		
	Dilute hydrochloric acid	Skin, eye, and respiratory irritation.	All these bulk reagents should be prepared in well-ventilated areas and dispensed using repipettors wherever practical.
	Phosphotungstic acid		
	Dilute acetic acid		
	Dilute phosphoric acid		
4. Disposal Procedures	Reagent waste	See above	Flush into waste disposal sink with large quantities of water.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standard

<i>Compound</i>	<i>Analytical Range (ppb)</i>	<i>Repeatability % CV</i>	<i>Reproducibility % CV</i>
Lactose (NFDM)	‡	< 15.0	< 20

‡ Limit may vary due to sample and aliquot sizes and sample type.

2. Critical Control Points and Specifications

<i>Record</i>	<i>Acceptable Control</i>
Yeast preparations	Prepare <i>exactly</i> as written. Activity checked using control sample containing NFDM.
pH 4.8 buffer solution	Check on pH meter before use. Calibrate meter with pH 4 or 5 buffer.
Benedict's solution	All weights of constituents are critical to ± 0.1 g. Must be filtered before use.
Iodine and thiosulfate standard solutions	1:1 (ratio) ± 0.1 g
Lactose standard solution	1.5789 g lactose diluted to 1 L.
Sample size	20 g ± 0.1 g.
Steam bath	All sample lumps must be broken up.
After steam bath	Must be cooled to room temperature before addition of dilute HCl.
Filter paper	Must be moistened before filtering, after phosphotungstic acid addition.
Neutralization	Proper pH depending on presence or absence of corn syrup or solids.
Yeast	Washed in absence of corn syrup; acclimated in presence of corn syrup.
Incubation temperature	30° C ± 1 ° C.
Incubation time	1 hr ± 5 min: washed yeast; 3 hr ± 5 min: acclimated yeast.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

Boiling with Benedict's solution	Cover with watch glass. Bring to boil in 4 ± 1 min. Boil for <i>exactly</i> 3 min.
After boiling	Cool in ice bath immediately.
Addition of acetic acid and iodine solutions	After addition of iodine, wait 5 min and check for complete dissolution of the copper oxide precipitate.
Addition of phosphoric acid	Carefully, with watch glass just ajar. Swirl to mix <i>slowly</i> . Keep watch glass on. Rinse underside of watch glass into flask with distilled water before titrating.
Titration	Calibrate buret at 10 mL intervals. Titrate slowly.
Calculation	Recheck.

3. Readiness To Perform

- a. Familiarization.
 - i. Phase I: Standards—3 replicates of ratios A and B.
 - ii. Phase II: Fortified samples—Replicates of previously analyzed samples.
- b. Acceptability criteria.
See section J.1 above.

4. Intralaboratory Check Samples

- a. System, minimum contents.
 - i. Frequency: Not to exceed 5% of samples.
 - ii. Blind samples or random replicates chosen by supervisor after initial analysis.
 - iii. Records are to be maintained by analyst and reviewed by the supervisor and Laboratory QA Officer.
- b. Acceptability criteria.
 - i. Stop all official analyses for that analyst.
 - ii. Investigate and identify probable cause.
 - iii. Take corrective action.
 - iv. Repeat Phase II of section J.3 if cause was analyst-related.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

**5. Sample Acceptability
and Stability**

-
- a. Matrix: Processed meat products.
 - b. Sample receipt size, minimum: 1 pound.
 - c. Condition upon receipt: Cold, sealed from air.
 - d. Sample storage.
 - i. Time: 1 week.
 - ii. Condition: Frozen.
-

6. Sensitivity

-
- a. Lowest detectable level (LDL): Not determined.
 - b. Lowest reliable quantitation (LRQ): 0.3%.
 - c. Minimum proficiency level (MPL): 0.3%.
-

NONFAT DRY MILK BY GLC

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DETERMINATIVE METHOD

A. INTRODUCTION

Theory

Nonfat dry milk is added to meat and poultry processed products as a binder. The amount is limited to 3.5% (in the absence of other binders) in products that have an added water requirement.

Lactose from the nonfat dry milk is extracted with distilled water on a steambath. The water is removed in a freeze dryer. The dry solid is silylated and quantitated by GLC, using a flame ionization detection. Lactose appears as two peaks (the d and l isomers) with retention times of approximately 4.65 and 6.35 min, using the GC parameters as stated in section B.

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

-
- a. Freeze dryer—Virtis Model 10—145 MRBD with Model 10-MCTR cabinet, or equivalent.
 - b. Scintillation vials (20 mL, w/screw cap).
 - c. Centrifuge—Sorvall Model RC-5, or equivalent.
 - d. Culture tubes—16 × 100 mm (Pyrex 9825).
 - e. Sugar flask, 100 mL (Kohlrausch)—(Pyrex 5780).
-

2. Instrumentation

-
- a. Gas Chromatograph—Tracor 222, or equivalent, equipped with a flame ionization detector. Operating parameters for the Tracor 222: injector temperature, 240° C; detector temperature, 250° C; column oven, 260° C. Gas flow rates: nitrogen, 40 mL/min; hydrogen, 45 mL/min; air, 400 mL/min.
 - b. GLC column—6 ft × 4 mm id, U-shaped; glass. Pack with 5% OV-1 on 100/120 mesh chromosorb W-HP. Condition at 280° C until stable baseline is obtained.
-

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List	<hr/>
--------------------------------------	-------

- | | |
|----|---|
| | <hr/> |
| a. | Pyridine, anhydrous, ACS reagent grade. |
| b. | Hexamethyldisilazane, Applied Science Laboratories, State College, PA 16801. |
| c. | Trimethylchlorosilane, Applied Science Laboratories, State College, PA 16801. |
| d. | Silylation reagent: Prepare a 4:2:1 mixture of reagents a., b., and c., respectively. |
| | <hr/> |

DETERMINATIVE METHOD

D. STANDARDS

**Preparation of
Standards**

Dilute 0.100 g of lactose to 100 mL with distilled water. Transfer 2.0 mL to a scintillation vial and remove the water by freeze drying. Add 2.0 mL of silylation reagent, shake vigorously, and let stand 1 hr.

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE

-
- | | |
|------------------------------|---|
| 1. Sample Preparation | <ul style="list-style-type: none">a. Weigh 10.0 g of ground meat into a 100 mL sugar (Kohlrusch) flask.b. Add ca 50 mL of water and mix to break up the lumps.c. Heat on a steambath for 30 min, mixing occasionally.d. Cool, dilute to volume, and filter.e. Pipet, 2.0 mL of filtrate into a scintillation vial and freeze dry. (This operation may proceed overnight).f. Add 2.0 mL of silylation reagent, close vial with plastic cap, and shake vigorously. Let stand for 1 hr to allow reaction to reach completion. Transfer to culture tubes.g. Centrifuge for 15 min @ 2000 rpm to settle the precipitate. |
|------------------------------|---|
-
- | | |
|-------------------------|--|
| 2. Determination | <p>Inject 2.0 to 5.0 μL of clear solution onto gas chromatographic column and compare response to that of the standard.</p> |
|-------------------------|--|
-

DETERMINATIVE METHOD

G. CALCULATIONS

Procedure

$$\% \text{ Lactose} = \frac{R_s}{R_{std}} \times \frac{V_{sd} \times C_{std}}{V_s} \times \frac{Df}{W} \times 100 =$$

R_s = response of sample (the sum of the two lactose peak heights or peak areas)

R_{std} = response of standard (the sum of the two lactose peak heights or areas)

V_s = volume of sample injected

V_{std} = volume of standard injected

C_{std} = concentration of standard (mg/mL)

Df = dilution factor = 100, if method followed as written

W = weight of sample = 10,000 mg, if method followed as written

% NFDM = % lactose \times 2

NOTE: The multiplication factor 2 is used for calculation of % NFDM based on 50% lactose in the NFDM.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Determination of NFDM (Lactose) in Meat by GLC.		
2. Required Protective Equipment	Safety glasses, plastic gloves, lab coat.		
3. Procedure Steps	<u>Reagents</u>	<u>Hazards</u>	<u>Recommended Safe Procedures</u>
	Silylation reagent: prepare a 4:2:1 mixture of reagents: (a) pyridine (b) hexamethyldisilazane and (c) trimethylchlorosilane, respectively	Silylation reagents may emit toxic fumes and may cause severe skin irritation.	Should be prepared and dispensed in an efficient fume hood. Keep away from any heat source. The wearing of protective gear cannot be stressed too strongly.
4. Disposal Procedures	Silylated filtrate	Mild irritant.	Flush down disposal sink with large amounts of water.

NIACIN/NIACINAMIDE (TOTAL)

Contents

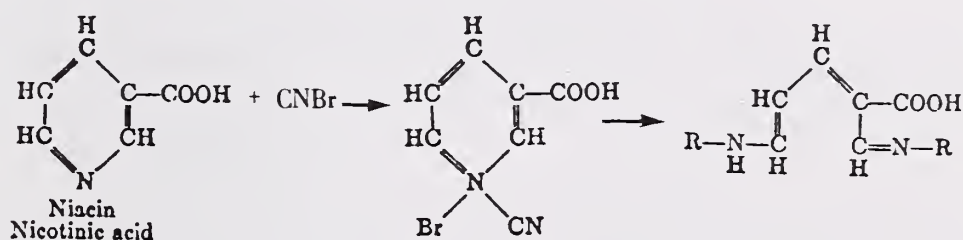
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DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory

Niacin reacts chemically the same as pyridine modified by a carboxyl group in the 3 position. Niacin reacts with cyanogen bromide to give a pyridinium compound that rearranges to form derivatives that will couple with aromatic amines, giving colored compounds.



2. Applicability

This method is applicable to the determination of total niacin or niacinamide in meat and poultry products.

Added niacin may be determined with method NIA2.

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

-
- a. Autoclave.
 - b. Volumetric pipettes: 5 mL, 40 mL.
 - c. Volumetric flask: 50 mL, 250 mL.
 - d. Glass syringe.
 - e. Erlenmeyer flasks: 1 L.
-

2. Instrumentation

Spectrophotometer suitable for reading at 450 nm.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and
Solution List

-
- a. Sulfuric acid, 1N: Dilute 27.8 mL conc. H_2SO_4 to 1 L.
 - b. Sodium hydroxide, 50% ACS reagent, or dissolve 50 g NaOH in 50 mL H_2O .
 - c. Bromocresol green indicator: Dissolve 0.1 g tetrabromo-m-cresolsulfophthalein in 3 mL 0.05N NaOH and dilute to 100 mL (yellow 3.8-5.4 blue).
 - d. Ammonium sulfate, $(\text{NH}_4)_2\text{SO}_4$: Reagent grade, crystal.
 - e. Ammonium hydroxide: Dilute 5 mL conc. NH_4OH to 250 mL.
 - f. Sulfanilic acid: Mix 10 g $\text{NH}_2\text{C}_6\text{H}_4\text{SO}_3\text{H}$ with 85 mL distilled water and dissolve by adding 1 mL increments of conc. NH_4OH . Adjust pH of solution to 4.5 with HCl and filter.
 - g. Hydrochloric acid: Mix 1 part HCl and 5 parts H_2O .
 - h. Cyanogen bromide solution: Weigh 50 g CNBr by difference into 2 L Erlenmeyer flask and dissolve in 450 mL warm (40°C) distilled water. Transfer to 500 mL volumetric flask, make to the mark, and store in a glass-stoppered bottle.

CAUTION: Use hood and rubber gloves!

NIA1
May, 1993

DETERMINATIVE METHOD

D. STANDARDS

Preparation of Standards

1 mg/mL nicotinic acid stock solution: Dissolve 0.05 g USP Nicotinic Acid Reference Standard in absolute ethanol, dilute to 500 mL, and refrigerate in low-actinic glass. Dilute 2 mL to 50 mL with water for standard solution: 1 mL = 4 μ g nicotinic acid.

DETERMINATIVE METHOD

F. ANALYTIC PROCEDURE

Determination

-
- a. Weigh 28.35 g (1 oz) of meat sample into a 1 L Erlenmeyer flask and add 200 mL 1N H₂SO₄.
 - b. Autoclave 30 min at 15 psi (121° C) and cool to room temperature.
 - c. Add 10 mL of sample solution to the 100 mL 50% NaOH and adjust to pH 4.5 with further dropwise additions, using pH meter or bromcresol green as external indicator.

NOTE: If product contains bran, hold at pH 13 for 15 min before adjusting to pH 4.5.

- d. Adjust volume to 250 mL and filter.
 - e. Pipet 40 mL filtrate into 50 mL volumetric flask containing 17 g ammonium sulfate. Mix, dilute to mark, and filter.
 - f. Pipet 40 mL standard (1 mL = 4 µg) into 50 mL volumetric flask containing 17 g ammonium sulfate. Mix and dilute to mark.
 - g. Use a pair of cuvettes for each sample and standard. Pipet 1 mL filtrate into each and add 5 mL distilled water to one of each pair (the blank).
 - h. Warm up spectrophotometer and set wavelength at 450 nm.
 - i. Develop each chromophore and read absorbance before proceeding with the next cuvette. Use blank to zero instrument; then read the sample.
 - j. To the blank, add 0.5 mL ammonium hydroxide (calibrated dropper) and mix; add 2 mL sulfanilic acid (pipette) and mix; and add 0.5 mL hydrochloric acid (calibrated dropper) and mix. Set instrument to zero absorbance within 2-3 min.
 - k. To the sample (or standard), add 0.5 mL ammonium hydroxide and mix, add 5 mL cyanogen bromide solution (all glass syringe) and mix, and let stand 30 sec. Add 2 mL sulfanilic acid and mix, add 0.5 mL hydrochloric acid and mix, and read absorbance within 2-3 min.
-

DETERMINATIVE METHOD

G. CALCULATIONS

1. Procedure

United States Recommended Daily Allowance (USRDA) = 20 mg.

$$\frac{A_{\text{sample}}}{A_{\text{std}}} \times \frac{35.27}{1000} \times F = \text{mg/serving}$$

$$\% \text{ USRDA} = \frac{\text{mg/serving}}{20} \times 1000$$

A_{sample} = absorbance of sample

A_{std} = absorbance of standard

$$35.27 = 3.2 \mu\text{g/mL} \times \frac{50 \text{ mL}}{40 \text{ mL}} \times \frac{250 \text{ mL}}{28.35 \text{ g}}$$

F = serving size converted to the appropriate dimensions (i.e., oz to g, etc.)

$$1000 = \mu\text{g per mg}$$

2. Reference

Methods of Vitamin Assay, The Association of Vitamin Chemists (1966).

NIACIN/NIACINAMIDE (ADDED)

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DETERMINATIVE METHOD

A. INTRODUCTION

Applicability

This procedure deals with added niacin, not total niacin. Method NIA1 should be used to determine total niacin (bound plus added). Due to the colloidal nature of nonfat dry milk, this method will not work quantitatively on that type of sample.

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

-
- a. Volumetric flasks: 100 mL, 50 mL.
 - b. Volumetric pipettes: 1 mL, 5 mL, 10 mL.
 - c. Funnel.
 - d. Filter paper.
-

2. Instrumentation

Spectrophotometer suitable to read at 450 nm.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and
Solution List

-
- a. Cyanogen bromide solution: (Caution: Use hood and rubber gloves.) Weigh 50 g CNBr by difference into 2 L Erlenmeyer flask and dissolve in 450 mL warm (40° C) distilled water. Transfer to 500 mL volumetric flask, make to the mark, and store in a glass stoppered bottle.
 - b. Aniline solution: Dilute 2.5 mL aniline to 100 mL with distilled water.
-

DETERMINATIVE METHOD

D. STANDARDS

**Preparation of
Standards**

Niacin stock solution: Dissolve 0.100 g U.S.P. niacin reference standard in absolute ethanol, dilute to 1 L with H₂O, and refrigerate in low-actinic glass. Dilute 2 mL to 50 mL with water for working standard solution (1 mL = 4 µg).

DETERMINATIVE METHOD

F. ANALYTIC PROCEDURE

1. Determination

- a. Dilute 5 g sample into 1 L volumetric flask with distilled water, mix well and filter.
- b. Pipet 10 mL aliquots (or other suitable aliquots) into two separate 50 mL volumetric flasks.
- c. Run niacin standard solution in parallel (2 mL = 8 μ g).
- d. Add 5 mL CNBr solution and 1 mL aniline solution to one sample and one standard flask. Dilute to volume with distilled water.
- e. Dilute the second sample flask and the second standard flask to volume with distilled water. These are the sample blank and the standard blank.
- f. Measure the absorbance at 450 nm, 20 min after the addition of reagents. Use the sample blank to zero the spectrophotometer.
- g. Read the absorbance of the sample solution.
- h. Re-zero the spectrometer with the standard blank.
- i. Read the absorbance of the standard solution.

NOTE: High protein samples, such as soya and mustard, tend to get hazy on standing. This haze is not filterable. Therefore, apparent high recoveries are obtained. In evaluating the results, disregard percentages below 0.1% even though this method can detect levels less than 0.1%.

2. Rapid Qualitative Test

Pour any convenient equal amounts of the initial sample dilution into separate beakers. To one add about 5 mL CNBr solution and 1 mL aniline solution; mix well. A positive test is an intense yellow color produced in this aliquot as opposed to the portion with no reagents added.

DETERMINATIVE METHOD

G. CALCULATIONS

Procedure

Compare standard and sample by proportion.

$$\text{ppm niacin} = \frac{\text{Abs}_{\text{sample}} (A)}{\text{Abs}_{\text{standard}} (B)}$$

A = μg niacin in standard aliquot.

B = Weight of sample in g.

Abs = Absorbance.

or

$$\% \text{ niacin} = \frac{\text{Abs}_{\text{sample}} (A)}{\text{Abs}_{\text{standard}} (B)} \times 100$$

A = μg niacin in standard aliquot.

B = Weight of sample in final aliquot in μg .

NOTE: The following values represent the average natural niacin content of fresh meat; analytical findings that more than double these values are to be considered in violation.

Beef 45 ppm

Pork 50 ppm

Veal 70 ppm

The levels of niacin in processed meats may be found in the "Composition of Foods," Agriculture Handbook, No. 8.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Niacin/Niacinamide (Added).		
2. Required Protective Equipment	Safety glasses, plastic gloves, lab coat.		
3. Procedure Steps		<u>Hazards</u>	<u>Recommended Safe Procedures</u>
	Cyanogen bromide solution.	Eye, skin, and respiratory irritation. Toxic effects similar to hydrogen cyanide.	Prepare and dispense in an efficient fume hood.
	Aniline solution.	Skin and respiratory irritation.	Same as above.
4. Disposal Procedures	Cyanogen bromide waste solution.	Cyanogen bromide	Store in labelled container and keep in well-ventilated area awaiting proper disposal.

NITRATES IN MEAT

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DETERMINATIVE METHOD

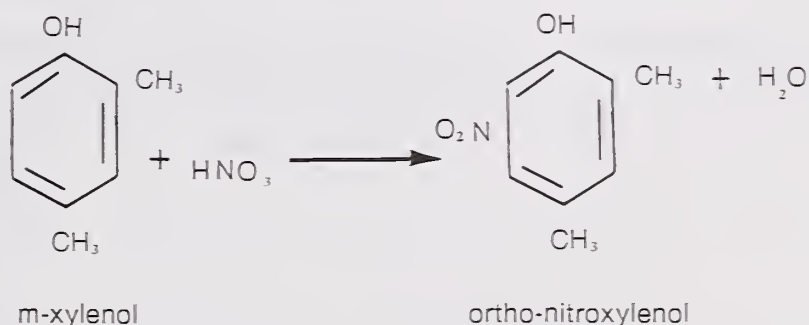
A. INTRODUCTION

Theory

In this method, nitrite and nitrate are determined as total nitrate, and the actual nitrate content calculated by difference. Therefore, a quantitative nitrite analysis must also be performed.

This procedure is based on the fact that nitrates yield nitric acid upon treatment with sulfuric acid. The nitric acid liberated is employed to nitrate a xyleneol. If, upon nitration, the incoming nitro group goes ortho to the hydroxyl group, the ortho-nitroxyleneol formed will be steam-distillable. The nitroxyleneol is then steam-distilled, and the distillate collected in an aqueous solution of NaOH, forming a colored sodium salt that follows Beer's law.

Because there are a number of isomers of xyleneol (dimethyl phenol), m-xyleneol was chosen as a reagent in this analysis as it will yield only one mono-nitro derivative upon nitration. Furthermore, the derivative will be ortho-nitroxyleneol due to the strong ortho-para orientation of the OH group.



The 6 position on the benzene ring is the one at which nitration occurs since the 2 and 4 positions (ortho and para to the OH group) are occupied by CH₃ radicals. Nitration at the 6 position forms ortho-nitroxyleneol that is steam-distillable.

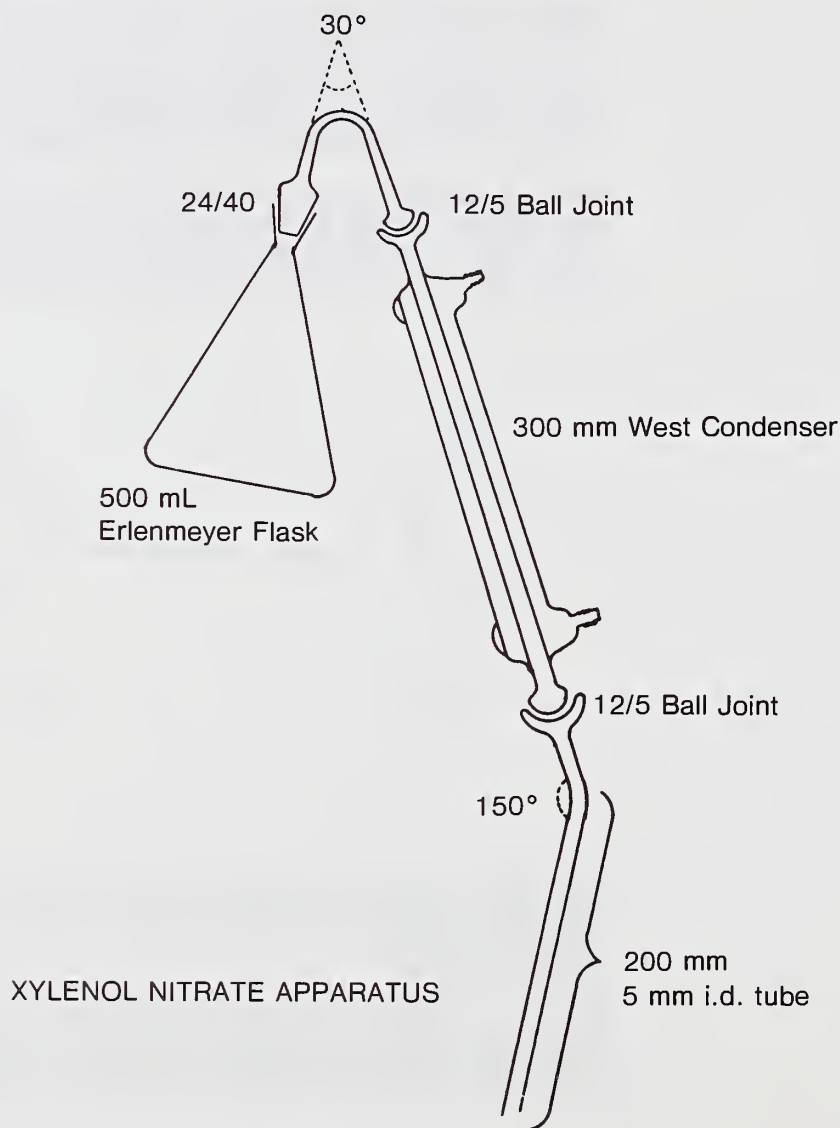
Nitrites, chlorides, and proteins interfere and must be removed. Nitrite is converted to nitrate by oxidation with KMnO₄, chlorides are precipitated as the silver salt, and proteins brought down with phosphotungstic acid.

DETERMINATIVE METHOD

B. EQUIPMENT

Apparatus

All-glass distillation apparatus with an elongated delivery tube. Available from Kontes and Ace by special order only.



NOTE: Kontes Glass
P.O. Box 729
Vineland, NJ 08360
Ph# 1-800-223-7150

Ace Glass Inc.
P.O. Box 688
1430 Northwest Blvd.
Vineland, NJ 08360
Ph# 1-800-543-6752
FAX# 609-692-3333

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and
Solution List

-
- a. Sulfuric acid: 1 volume concentrated reagent grade H_2SO_4 + 10 volumes H_2O . (1 + 10)
 - b. Sulfuric acid: 3 volumes concentrated reagent grade H_2SO_4 + 1 volume H_2O . (3 + 1)
 - c. Potassium permanganate 0.2N: Dissolve 0.63 g KMnO_4 in H_2O and dilute to 100 mL.
 - d. Phosphotungstic acid solution: 20% w/v.
 - e. Silver-ammonium hydroxide solution ($\text{Ag} - \text{NH}_4\text{OH}$): Dissolve 5 g nitrate-free Ag_2SO_4 in 60 mL NH_4OH . Heat to boiling, concentrate to approximately 30 mL, cool, and dilute to 100 mL with H_2O .
 - f. m-Xylenol: Aldrich #D17,460-2 (2, 4-dimethylphenol).
 - g. Sodium hydroxide solution: 1% w/v.
 - h. Bromocresol green indicator: Dissolve 0.1 g bromocresol green in 1.5 mL 0.1N NaOH and dilute to 100 mL with H_2O .
-

DETERMINATIVE METHOD

D. STANDARDS

- | | |
|-------------------|---|
| 1. Source | Sodium nitrate—Aldrich #22,993-8. |
| <hr/> | |
| 2. Standard Curve | <ul style="list-style-type: none">a. Dissolve 0.1600 g dried reagent-grade NaNO_3 in distilled H_2O and dilute to 1 L. Each mL of this solution contains 160 μg of NaNO_3.b. Pipet 0, 1.0, 2.5, 7.5, and 10.0 mL of this solution into separate 500 mL Erlenmeyer flasks and add a volume of distilled H_2O to each such that a 10 mL volume is obtained.c. Continue as in section F.1.h. "Add 45 mL H_2SO_4 (3+1), stopper flask, mix..." Plot absorbance 445 nm vs. $\mu\text{g NaNO}_3/\text{mL}$. |

NOTE: The standard curve as prepared here covers NaNO_3 concentrations up to 2,000 ppm for a 10g sample diluted as stated in section F. For better measurements at low concentrations (< 50 ppm), prepare a curve using standards that bracket the concentration of the sample, that is, one-half to twice the level of interest. For low concentration, use 5 cm cuvettes.

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE

1. Determination

- a. Weigh 10.0 g sample into a 100 mL sugar flask. Add 80 mL distilled H_2O , stir or shake to break up any lumps, and heat on steam bath for 1 hr.
- b. Cool, dilute to volume using bottom of fat layer as meniscus, mix well, and filter.
- c. Pipet 40 mL filtrate into a 50 mL volumetric flask. At this point, add 40 mL of distilled H_2O to a 50 mL volumetric flask, and carry through the procedure as a reagent blank.
- d. Add 3 drops of bromocresol green indicator and dilute H_2SO_4 (1 + 10) dropwise until color changes to yellow.
- e. To assure complete oxidation of nitrite to nitrate, add 0.2N KMnO_4 dropwise with shaking until permanent pink color is obtained.
- f. Add 1 mL dilute H_2SO_4 (1 + 10) and 1 mL phosphotungstic acid solution, dilute to mark with H_2O , mix well, and filter.
- g. Pipet 10 mL filtrate into a 500 mL Erlenmeyer flask and add sufficient $\text{Ag-NH}_4\text{OH}$ solution dropwise to precipitate all chlorides and most of the excess phosphotungstic acid. (A slight excess of the Ag reagent is not harmful.) Placing the flask upon a black surface will help determine the end point.
- h. Add 45 mL H_2SO_4 (3 + 1), stopper flask, mix, cool to approximately 35°C , add 3 drops of m-xyleneol, stopper, mix thoroughly, and maintain at $30^\circ\text{-}40^\circ\text{C}$ for 30 min.
- i. After nitration is complete, add 150 mL H_2O (wash stopper) and a few glass beads, and distill 40-50 mL into a 100 mL volumetric flask, placed in a beaker of water and ice, containing 5 mL of 1% NaOH solution and sufficient H_2O such that the end of the delivery tube will be submerged. Remove any nitroxyleneol that may have solidified in the condenser by stopping condenser water flow and allowing condenser to become hot. Do not allow steam to bubble through the distillate.
- j. Dilute to volume with H_2O and measure absorbance at 445 nm, setting the spectrophotometer at 100% transmission with reagent blank. Obtain NaNO_3 concentration from standard curve and calculate total nitrate content.

DETERMINATIVE METHOD

G. CALCULATIONS

1. Procedure

$$\text{Total NaNO}_3 \text{ content (ppm)} = \frac{(A)(B)}{C}$$

A = $\mu\text{g NaNO}_3/\text{mL}$ (from standard curve).

B = Final volume of distillate (100 mL if determination is conducted as written above).

C = Sample weight in grams represented in final volume of distillate (0.8 g if dilutions are made as written above).

In order to obtain nitrate content (due solely to NaNO_3), multiply (if present) the sodium nitrite content (ppm) by the ratio

$$\frac{\text{NaNO}_3}{\text{NaNO}_2} = \frac{85}{69} = 1.23$$

and subtract this product from the total nitrate content.

$$\text{NaNO}_3 \text{ content (ppm)} = \text{Total NaNO}_3 \text{ content} - (1.23)(\text{NaNO}_2 \text{ content}).$$

If nitrate is present as potassium nitrate, multiply NaNO_3 content by the ratio

$$\frac{\text{KNO}_3}{\text{NaNO}_3} = \frac{101}{85} = 1.19$$

$$\text{KNO}_3 \text{ content (ppm)} = (1.19)(\text{NaNO}_3 \text{ content}).$$

2. Reference

Official Methods of Analysis of the Association of Official Analytical Chemists, 15th Edition, 935.48.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Nitrates in Meat		
2. Protective Equipment	Safety glasses, plastic gloves, heat resistant gloves, face shield, and lab coat.		
3. Procedure Steps		<u>Hazards</u>	<u>Recommended Safe Procedures</u>
	C. Reagents		
	b. Sulfuric acid (3 + 1)	Danger of spattering severe skin and respiratory irritant	Slowly add acid to water that is sitting in an ice bath. Stir while adding.
	c. Potassium permanganate	Skin irritant and fire hazard	All these reagents should be prepared in a fume hood, making maximum use of protective equipment.
	e. Silver-ammonium hydroxide	Severe skin and respiratory irritant	
	F. Analytic Procedure		
	h. Add 45 mL H ₂ SO ₄ (3 + 1), stopper, mix, cool to approx. 35° C, add 3 drops of m-xyleneol, etc.	H ₂ SO ₄ can cause severe skin and respiratory irritation. m-Xyleneol is a toxin that can be readily absorbed through the skin.	As stated above, protective equipment and fume hood must be used to the fullest extent.
4. Disposal Procedures	H ₂ SO ₄ and NaOH solutions.	See above	Solutions can be flushed down a disposal sink with large amounts of water.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standard

<i>Compound</i>	<i>Analytical Range (ppm)</i>	<i>Repeatability % CV</i>	<i>Reproducibility % CV</i>
Nitrates in Meat	‡	ND	ND

‡ Limit may vary due to sample and aliquot sizes and sample type.

2. Critical Control Points and Specifications

<i>Record</i>	<i>Acceptable Control</i>
Distilling apparatus	Exactly as described.
Sample size	10.0 g \pm 0.1 g.
pH indicator	Bromcresol green or calibrated pH meter; 3.8 pH \pm 0.1.
Complete chloride precipitation	Check for precipitation by adding silver ammonium hydroxide dropwise with flask on black surface. Continue until no further precipitation occurs.
Cooling temperature	35° C \pm 2° C.
Incubation temperature and time	35° C \pm 5° C for 30 min.
Distillation	Must use exact apparatus as shown in section B. Distill 45 \pm 5 mL. With condenser warm, continue to distill an additional 10 mL to remove any solidified nitroxylenol.
Spectrophotometry	Zero instrument with reagent blank.
Standard curve	Weight of dried sodium nitrate (2 hr at 100° C \pm 2° C) = 0.1600 g \pm 0.0002 g. Each analyst must prepare his/her own curve. At least a single point must be checked with each day's samples. The absorbance of that one point must agree within \pm 5% of the absorbance obtained on the original curve; for example, 0.200 \pm 0.010 absorbance units. A new curve must be prepared with each new bottle of m-xyleneol and for each spectrophotometer used. Linear regression equations may be used.
Calculations	Recheck.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

3. Readiness To Perform

a. Familiarization.

- i. Phase I: Standards—Prepare standard curve.
- ii. Phase II: Fortified samples—Random replicates selected by supervisor.

b. Acceptability criteria.

See section J.1 above.

4. Intralaboratory Check Samples

a. System, minimum contents.

- i. Frequency: Not to exceed 20% of samples.
- ii. Blind samples or random replicates chosen by supervisor after initial analysis.
- iii. Records are to be maintained by analyst and reviewed by the supervisor and Laboratory QA Officer.

b. Acceptability criteria.

If unacceptable values are obtained, then:

- i. Stop all official analyses for that analyst.
 - ii. Investigate and identify probable cause.
 - iii. Take corrective action.
 - iv. Repeat Phase II if cause was analyst-related.
-

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

**5. Sample Acceptability
and Stability**

-
- a. Matrix: Cured meat products.
 - b. Sample receipt size, minimum: 500 g.
 - c. Condition upon receipt: Frozen.
 - d. Sample storage.
 - i. Time: 1 week.
 - ii. Condition: Frozen.
-

6. Sensitivity

-
- a. Lowest reliable quantitation (LRQ): 25 ppm.
 - b. Minimum proficiency level (MPL): 25 ppm.
-

NITRATES IN CURES AND PICKLES

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DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory

In this method, nitrite and nitrate are determined as total nitrate by the Devarda procedure, and the nitrate content calculated by the difference. When Devarda alloy (50% Cu, 45% Al, 5% Zn) reacts with NaOH, nascent hydrogen is liberated. Nascent hydrogen will reduce nitrites and nitrates quantitatively to ammonia. The ammonia is collected in an excess of standard acid solution, and the excess acid titrated with standard alkali solution.

The distillation is performed until the volume of the mixture in the distillation flask is quite low, a relatively high concentration of NaOH being required for complete reduction of nitrites and nitrates to ammonia.

2. Applicability

This procedure is not applicable in the presence of nitrogenous organic matter.

DETERMINATIVE METHOD

B. EQUIPMENT

Apparatus

-
- a. Kjeldahl distillation equipment.
 - b. Kjeldahl flask: 800 mL.
 - c. Erlenmeyer flask: 500 mL.
 - d. Titration apparatus.
-

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and
Solution List

-
- a. Devarda alloy.
 - b. NaOH solution: 50% (w/w).
 - c. Standard NaOH solution: $0.2000 \pm 0.0004N$. Add 108 mL of (1 + 1) NaOH to CO_2 -free distilled H_2O and dilute to 10 L. Standardize against potassium acid phthalate, using phenolphthalein indicator.
 - d. Indicator solution: Fleisher methyl purple.
 - e. Carnauba wax or other suitable anti-foaming preparation.
-

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE

Determination

-
- a. To a 500 mL Erlenmeyer flask, add 25.0 mL of 0.2000N HCl or H₂SO₄, methyl purple indicator, and sufficient distilled H₂O to submerge the end of the delivery tube. Place the flask in position on the distillation assembly.
 - b. Weigh 20.0 g of dry cure into a 200 mL volumetric flask.
 - c. Add 100 mL distilled H₂O, shake to dissolve cure, dilute to volume with distilled H₂O, stopper, and mix well.
 - d. Transfer a 20 mL aliquot to an 800 mL Kjeldahl flask. [For pickles, transfer 25.0 mL sample (making no dilutions) to an 800 mL Kjeldahl flask.]
 - e. Add 275 mL distilled H₂O, 3 g of Devarda alloy, a small quantity of anti-foaming material, and 5 mL of 50% NaOH solution, pouring the latter down the side of the flask so that it does not immediately mix with the contents.
 - f. Place a glass wool plug in the neck of the flask. Connect the flask to the distilling bulb on the condenser, rotate the flask to mix the contents thoroughly, and place the flask on the heater.
 - g. Heat slowly at first, and then at a rate that will yield 250 mL of distillate in 1 hr.
 - h. Collect the 250 mL of distillate and titrate the excess standard acid with standard NaOH.
-

DETERMINATIVE METHOD

G. CALCULATIONS

1. Procedure (Cures)

$$\% \text{ total NaNO}_3 = \frac{(V_A N_A - V_B N_B) 8.5}{S}$$

where

V_A = Volume of standard acid added

V_B = Volume of standard base used in titration

8.5 = Meq. of NaNO_3 (includes factor of 100 for percentage)

N_A = Normality of standard acid

N_B = Normality of standard base

S = Sample weight in grams represented by aliquot in Kjeldahl flask.

If both the acid and base are 0.2000N, the equation reduces to:

$$\% \text{ total NaNO}_3 = \frac{(V_A - V_B)(1.7)}{S}$$

In order to obtain the % NaNO_3 (due solely to NaNO_3), multiply the sodium nitrite content (%) (if present) by the ratio $\text{NaNO}_3/\text{NaNO}_2 = 85/69 = 1.23$, and subtract this product from the % total NaNO_3 .

$$\% \text{ NaNO}_3 = \% \text{ total NaNO}_3 - 1.23 (\% \text{ total NaNO}_2)$$

If the nitrate is present as KNO_3 , multiply % NaNO_3 by the ratio $\text{KNO}_3/\text{NaNO}_3 = 101/85 = 1.19$

$$\% \text{ KNO}_3 = (1.19) (\% \text{ NaNO}_3)$$

2. Procedure (Pickles)

Because the amount of nitrate that may be added to pickles is measured in pounds nitrate per 100 gal of pickles, the result should bear the same unit. First express the result in g NaNO_3 per mL of pickle, and proceed from there.

$$\text{Total NaNO}_3 \text{ content (g/mL)} = \frac{(V_A N_A - V_B N_B) 0.085}{V_p}$$

where

V_A = Volume of standard acid added

V_B = Volume of standard base used in titration

0.085 = Meq. wt. of NaNO_3

N_A = Normality of standard acid

N_B = Normality of standard base

V_p = Volume of pickle

DETERMINATIVE METHOD

G. CALCULATIONS (Continued)

If both the acid and base are 0.2000N, the equation becomes:

$$\text{Total NaNO}_3 \text{ content (g/mL)} = \frac{(V_A - V_B)(0.017)}{V_P}$$

To convert g/mL to lb/100 gal, it is only necessary to multiply the former by 834.5.

To obtain the NaNO₃ content (due solely to NaNO₃, multiply the sodium nitrite (if present) contents (lb/100 gal) by the ratio NaNO₃/NaNO₂ = 85/69 = 1.23, and subtract this product from the total NaNO₃ content.

$$\text{NaNO}_3 \text{ content (lb/100 gal)} = \text{total NaNO}_3 \text{ content} - (1.23)(\text{NaNO}_2 \text{ content}).$$

If the nitrate is present as KNO₃, multiply NaNO₃ content by the ratio KNO₃/NaNO₃ = 101/85 = 1.19.

$$\text{KNO}_3 \text{ content (lb/100 gal)} = (1.19)(\text{NaNO}_3 \text{ content}).$$

3. Reference

Official Methods of Analysis of the Association of Official Analytical Chemists, 15th Edition.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Nitrate in Cures and Pickles		
2. Protective Equipment	Safety glasses, heat resistant gloves, face shield, and lab coat.		
3. Procedure Steps		<u>Hazards</u>	<u>Recommended Safe Procedures</u>
	C. Reagents		
	b. NaOH solution 50% (w/w)	Irreversible tissue damage	Obey protective equipment requirements without exception.
	F. Determination		
	This is essentially a Kjeldahl procedure with the mercury hazard eliminated. Refer to the PRO1 method.		
4. Disposal Procedures	Aqueous reaction products	Skin and respiratory irritant.	Flush into disposal sink with large amounts of water.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standard

<i>Compound</i>	<i>Analytical Range % percent</i>	<i>Repeatability % CV</i>	<i>Reproducibility % CV</i>
Nitrates in Cures and Pickles	‡	ND	ND

‡ Limit may vary due to sample and aliquot sizes and sample type.

2. Critical Control Points and Specifications

<i>Record</i>	<i>Acceptable Control</i>
Receiving flask	25 mL 0.2000N acid \pm 0.0004N by pipet.
Sample size	20.00 g \pm 0.05 g for dry cures. 20.0 mL by pipet for pickles.
Devarda alloy	3.0 g \pm 0.1 g.
50% sodium hydroxide	5.0 mL \pm 0.5 mL.
Distillation	Start slowly. Collect 250 mL \pm 5 mL.
Calculations	Recheck.

3. Readiness To Perform

- a. Familiarization.
 - i. Phase I: Standard—Standardize against potassium acid phthalate.
 - ii. Phase II: Fortified samples
 - iii. Phase III: Check samples for analyst accreditation.
- b. Acceptability criteria.
See section J.1 above.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

4. Intralaboratory Check Samples

- a. System, minimum contents.
 - i. Frequency: Not to exceed 5% of sample.
 - ii. Blind samples or random replicates chosen by supervisor after initial analysis.
 - iii. Records are to be maintained by analyst and reviewed by the supervisor and Laboratory QA Officer.
 - b. Acceptability criteria.

If unacceptable results are obtained, then:

 - i. Stop all official analyses for that analyst.
 - ii. Investigate and identify probable cause.
 - iii. Take corrective action.
 - iv. Repeat Phase III of section J.3 if cause was analyst-related.
-

5. Sample Acceptability and Stability

- a. Matrix: Pickles and dry cure.
 - b. Sample receipt size, minimum: 500 mL pickles or 1 lb cure.
 - c. Condition upon receipt: 4° C.
 - d. Sample storage:
 - i. Time: 1 week.
 - ii. Condition: Refrigerate 4° C.
-

6. Sensitivity

- a. Lowest detectable level (LDL): Not determined.
 - b. Lowest reliable quantitation (LRQ): 0.1 %
 - c. Minimum proficiency level (MPL): 0.1 %
-

NITROGEN/AUTOMATED

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DETERMINATIVE METHOD

A. INTRODUCTION

Theory

NH_3 reacts with hypochlorite and phenate ion in alkaline solution to produce quinonechloramine which reacts with additional phenate ion, producing the blue dissociated form of indophenol with maximum absorbance at 630 nm in alkaline solution.

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

-
- a. Pipet: Automatic zeroing, 50 mL (Kontes Glass Co., K-763280), or equivalent.
 - b. Tubing: Fluran 1'-5000, 0.125" id, Acidflex, or Teflon, 0.133" id.
 - c. Pipetting machine: Automatic Model 60453 with Model 70327 valve syringe (BBL, Division of BioQuest).
 - d. Laboratory mill (Straub Co., Croydon, PA 19020), Model 4-E, or equivalent.
 - e. Top-loading balance.
 - f. Volumetric flasks: 1 L, 2 L, and 3 L.
 - g. 1.5 L beakers.
 - h. 200 mL tall-form beaker.
 - i. Teflon-coated stirring bars.
 - j. Watch glass, 60 mm diameter.
-

2. Instrumentation

Automatic analyzer. AutoAnalyzer with following modules (Technicon Instruments Corp.): Sampler II; proportioning pump I; continuous digester; proportioning pump II; current stabilizer; constant temperature bath equipped with variable temperature regulator (set at 70° C); colorimeter with 15 mm tubular flowcell, 630 nm filters, and No. 9 aperture; voltage stabilizer; recorder with transmittance paper; vacuum pump; 2 manifolds (Figures 1 and 2, facing page); and 8.5 mL sample cups.

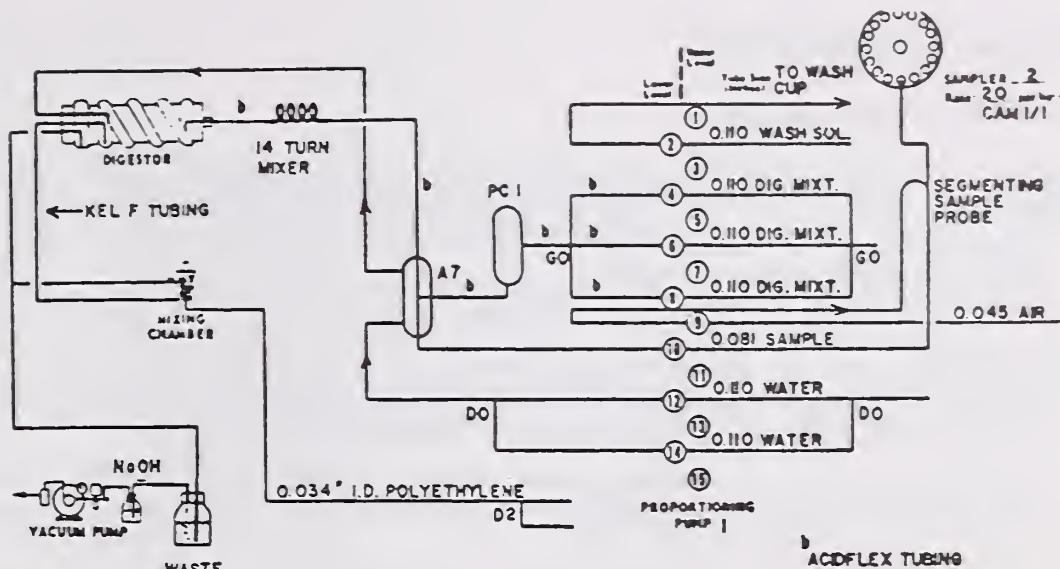


Figure 1.—Helix inlet manifold.

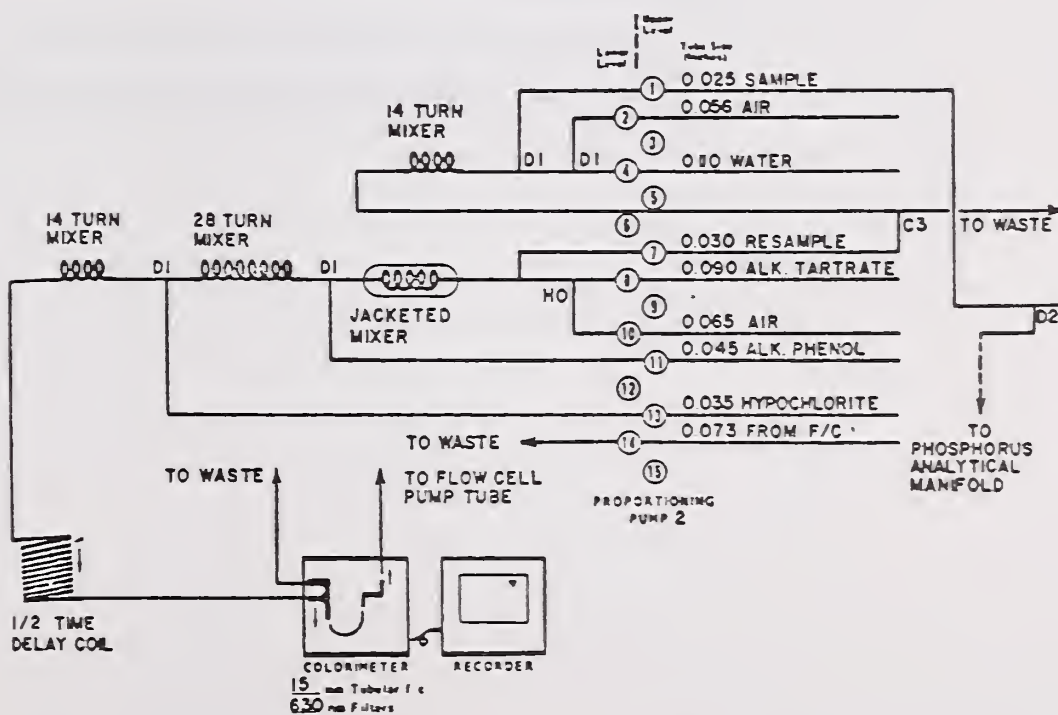


Figure 2.—Nitrogen analytical manifold.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

**Reagent and
Solution List**

-
- a. Vanadium pentoxide solution: Weigh 40.0 g NaOH pellets and transfer to 1 L volumetric flask. Add 500 mL H₂O, dissolve, and cool to room temperature. Add 12.5 g V₂O₅ to flask, dissolve, dilute to volume, and mix.
 - b. Digestion mixture: Caution—Mix in order 150 mL V₂O₅ solution, 90 mL 60-62% HClO₄, and 3460 mL H₂SO₄. Rate of consumption is 497 mL/hr.
 - c. Wash solution—H₂SO₄ (1 + 1). Caution: To 1 L H₂O in 2 L volume flask, add 1 L H₂SO₄ slowly with swirling. Cool to room temperature, dilute to volume with H₂O. Rate of consumption is 234 mL/hr.
 - d. Alkaline tartrate solution: Dissolve 150 g potassium sodium tartrate•4H₂O in 1950 mL H₂O and add 1050 mL 50% NaOH solution. Consumption rate is 174 mL/hr.
 - e. Sodium hypochlorite solution: 4 to 6% NaOCl (Fisher Scientific Co.) Consumption rate is 25 mL/hr.
 - f. Alkaline phenol solution: Prepare 15N NaOH solution by adding 2400 mL 50% NaOH (w/w) to 600 mL H₂O, cooling, and storing in polyethylene bottle. To 500 mL 15N NaOH in vessel cooled by circulating cold H₂O, slowly add 276 mL 90% liquid phenol. Cool to room temperature and dilute with water to 1 L. Store in dark in polyethylene bottle. Consumption rate is 48 mL/hr.
 - g. Dilution water.
 - i. Input manifold: Consumption rate is 468 mL/hr.
 - ii. Analytical manifold: Consumption rate is 234 mL/hr.
-

DETERMINATIVE METHOD

D. STANDARDS

1. Source	Wilson Certified Foods, Inc., P.O. Box 7345, Omaha, NE 68107.
2. Preparation of Standards	Grind freeze-dried beef four times in standard laboratory mill. Determine Kjeldahl nitrogen using 0.3-0.5 g. Based on nitrogen content, prepare standards containing 0.8, 1.2, 1.6, 2.0, 2.4, 2.8, 3.2, 3.6, and 4.0 mg N/mL as follows: Into 9 separate 1.5 L beakers, transfer weighed amount freeze-dried beef. Add 400 mL H ₂ O and disperse thoroughly, using magnetic stirrer. Slowly add, with stirring, 500 mL H ₂ SO ₄ and continue stirring 15 min. Cool in ice bath until fat solidifies. With aid of stirring rod and funnel, transfer solution through glass wool pad into 1 L volume flask. Let warm to room temperature, dilute with water to volume, and mix.
3. Storage Conditions	Store ground material in freezer to prevent deterioration. Store solution in polyethylene bottle.
4. Shelf Life Stability	Solution is stable up to 75 days.

NTG
May, 1993

DETERMINATIVE METHOD

E. SAMPLE HANDLING

Sample Preparation

Accurately weigh 10.00 g sample into 200 mL tall-form beaker. Pipet 53 mL H_2O into beaker with pipetting machine. Add 1" Teflon-coated stirring bar, cover with 60 mm watch glass, and disperse sample, using magnetic stirrer. With stirring, add 50 mL H_2SO_4 using automatic pipet, and continue stirring until sample is dissolved. Cool to room temperature in cooling bath.

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE

1. Analytical System

Use standard 0.0625" id transmission tubing throughout system unless otherwise specified. Pump sample at 2.5 mL/min and segment with air pumped at 0.8 mL/min. Pump digestion mixture at 8.28 mL/min through PCI fitting and add to sample at A7 fitting. Pass sample stream through 14-turn mixing coil into inlet of digester helix. Aspirate diluted sample into bubble chamber and remove aliquot for analysis at rate of 0.23 mL/min. Dilute aliquot with H₂O pumped at 3.90 mL/min and segment with air at 1.20 mL/min. Pass stream through 14-turn mixer and C3 debubbler, and resample at 0.32 mL/min. Add alkaline tartrate solution at 2.90 mL/min and air at 1.60 mL/min. Then pass stream through jacketed mixer, add alkaline phenol solution at 0.80 mL/min, and pass through double mixer. Add NaOCl solution at 0.42 mL/min and pass stream through 14-turn mixer and ½ time-delay coil for color development. Finally pass stream into colorimeter with 630 nm filter and 15 mm tubular flowcell into waste at 2.00 mL/min.

2. Start-Up Procedure

Place all reagent lines, except Acidflex, in water; turn on both proportioning pumps and digester power. Turn on vacuum pump, setting gauge at 12-15 psi. Pump digestion mixture and all analytical reagents through their respective lines to determine that system is operating properly. Prior to routine use, optimize digester unit as follows: Using 2.0 mg N/mL standard in duplicate, vary amperage setting according to following table, and record absorbance. Allow 20 min interval after changing setting to stabilize helix temperature before standard is analyzed. Use settings giving highest absorbance.

<u>Amperage Settings</u>		<u>Amperage Settings</u>	
Stage 1	Stages 2 and 3	Stage 1	Stages 2 and 3
2.50	3.00	4.20	6.40
3.00	4.00	4.50	7.00
3.50	5.00	5.00	8.00
3.80	5.60	5.50	9.00
4.00	6.00		

Set digester helix to rotate at 6.7 rpm, referring to Technicon Manual T-69-123 (1970) for instructions.

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE (Continued)

3. Determination

- a. Pour standard and prepared samples into 8.5 mL cups and place in Sampler II turntable.
- b. Adjust sampling rate to 20/hr, with 1:1 sample-to-wash ratio to provide 1.5 min sampling and 1.5 min wash.
- c. Press reset button and activate sampler turntable, thus passing standards and samples into analytical system. Place stop bar in turntable. (Formation of excessive fat deposits in sample line between segmenting sample probe and input manifold can be retarded by passing wash solution through double mixer wrapped with heating tape and covered with layer of aluminum foil and layer of asbestos; adjust temperature to 60° C with variable transformer connected to heating tape.)
- d. Read absorbance of samples from recorder chart and compare with standard curve of absorbance against mg N/mL. Include standard curve with every 30 samples.
- e. As 53 mL H₂O + 50 mL H₂SO₄ added to samples gives 95 mL (8 mL contraction), it may be assumed that 10 g samples containing 50% H₂O give final volume of 100 mL.
- f. However, certain dry products (e.g., pepperoni) or wet products (e.g., corned beef brisket) may contain considerably more or less than 50%, causing an error by as much as 0.6% protein. Close approximation may be obtained by adding H₂O content of sample, as determined in MOI method, to 95 mL to obtain final total volume. Using this assumption,

$$\% \text{ N} = \frac{[(\% \text{H}_2\text{O in sample } 10) + 95]}{\times (\text{mg N/mL})} \times 0.01$$

$$\% \text{ Protein} = \% \text{ N} \times 6.25$$

4. Shut-Down Procedure

Turn off heat switch and let first stage temperature reach 200° C. Remove helix cover and place all reagent lines except digestion mixture in H₂O after first stage temperature is 150° C. Place digestion mixture line in empty Erlenmeyer and let Acidflex pump tubes "air-wash." Rinse entire system for 15 min. Shut off proportioning pumps and break vacuum in liquid waste bottle. Turn off digester power switch and replace helix cover.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Automated Determination of Nitrogen.		
2. Required Protective Equipment	Safety glasses, face shield, heat-resistant gloves, plastic gloves, lab coat, and safety shoes.		
3. Procedure Steps	<u>Reagents</u>	<u>Hazards</u>	<u>Recommended Safe Procedures</u>
	a. Digestion mixture:— ext.	Harmful fumes, corrosive substances, severe skin and respiratory irritation can result.	It is strongly suggested that these reagents be prepared and used in area with exceptional air flow and environmental controls. Protective equipment should be emphasized.
	b. Wash solution— H ₂ SO ₄ (1 + 1)—etc.		
	c. Preparation of sample	Harmful fumes and thermal burns from conc. acid.	The analyst should resist the tendency to <i>rush</i> through this phase, thereby increasing the chances of accidents occurring.
	d. Start-up and shut-down procedures	Ruptured reagent lines and resultant spillage.	Procedures should be followed closely as outlined in the technician manual.
4. Disposal Procedures	Excess meat/acid digestion mixture. Reagent waste	Harmful fumes and thermal burns.	These liquids should be flushed with large quantities of water into an acid-resistant disposal sink. The area should be well ventilated.

NITRITES IN MEAT

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DETERMINATIVE METHOD

A. INTRODUCTION

Theory

In this procedure, residual nitrite (which has not combined to form nitrosomyoglobin) is determined spectrophotometrically. An aromatic primary amine will react with an acidified solution of a nitrite to produce a diazonium salt. If this salt is then condensed or coupled with another primary aromatic amine, an aminoazo compound is formed that obeys Beer's Law.

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

-
- a. pH meter capable of measuring 0.1 pH unit increments.
 - b. Balance sensitive to 0.1 mg increments.
 - c. Volumetric labware: pipets, flasks, etc.
 - d. Steam bath.
-

2. Instrumentation

Spectrophotometer capable of reading cells of 1-5 cm path length.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and
Solution List

-
- a. Sulfanilamide solution: Dissolve 0.5 g in 150 mL of 15% (v/v) HOAc.
 - b. N-(1-naphthyl) ethylenediamine dihydrochloride solution (NED): Dissolve 0.2 g in 150 mL 15% (v/v) HOAc. Store in g-s brown glass bottle.
 - c. Filter paper: Check 3-4 sheets randomly selected filter paper for nitrite contamination. Filter ca. 40 mL of H₂O through each sheet and add reagents. If positive, discard box.
 - d. Sodium hydroxide: 0.1N.
-

DETERMINATIVE METHOD

D. STANDARDS

1. Source

Sodium nitrite—Aldrich #23,721-2.

2. Standard Curve

- a. Dissolve 0.2 g dried NaNO_2 (take into account the assay of your NaNO_2 reagent) in distilled H_2O , dilute to 1 L, and mix well.
- b. Dilute 10.0 mL of this solution to 1 L with distilled H_2O and mix well. Each mL of the final dilution contains $2.0 \mu\text{g}$ NaNO_2 .
- c. Pipet 0.0, 5.0, 10.0, 20.0, and 25.0 mL of the final dilution into separate 50 mL volumetric flasks, add 2.5 mL of sulfanilamide solution, and mix by swirling.
- d. Allow to stand 5 min, add 2.5 mL of N-(1-naphthyl) ethylenediamine dihydrochloride solution, dilute to volume with distilled H_2O , and mix well.
- e. Let stand for 15 min and measure absorbance at 540 nm, setting the spectrophotometer at 0.0 absorbance with the reagent blank solution prepared above. Plot absorbance vs micrograms NaNO_2 per mL.

NOTE: The standard curve, as prepared above, covers NaNO_2 concentrations up to 200 ppm for a 5 g sample diluted as stated in section F. For better measurements at low concentration (less than 50 ppm), prepare a curve using standards that cover the concentration of the sample, that is one-half to twice the level of interest. For low concentrations use 5 cm cuvettes.

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE

Determination

-
- a. Weigh 5.0 g sample into a 400-600 mL beaker, add 300 mL of distilled H₂O, and stir vigorously to break up any lumps. (For acid products, adjust pH, with pH meter, to 6.5-7.0 with 0.1N NaOH.) Heat on steam bath for 2 hr with occasional stirring. Quantitatively transfer to a 500 mL volumetric flask.
 - b. Cool to room temperature, dilute to mark with distilled H₂O, mix well, and filter.
 - c. Transfer 25.0 mL of filtrate (or other suitable aliquot containing 5-50 μ g NaNO₂) to a 50 mL volumetric flask, add 2.5 mL of sulfanilamide solution, and mix by swirling.
 - d. Allow to stand for 5 min, add 2.5 mL of N-(1-naphthyl) ethylenediamine dihydrochloride solution, dilute to volume with distilled water, and mix well.
 - e. Let stand for 15 min, transfer a portion to a cuvette, and measure absorbance at 540 nm, setting the spectrophotometer at 0.0 absorbance with a reagent blank. Obtain NaNO₂ concentration from standard curve and calculate NaNO₂ content.
-

DETERMINATIVE METHOD

G. CALCULATIONS

1. Procedure

$$\text{NaNO}_2 \text{ content (ppm)} = \frac{(A)(B)}{C}$$

Where

A = Micrograms NaNO_2 per mL (from standard curve).

B = Final volume to which an aliquot was diluted (50 mL if conducted as written above).

C = Sample weight in grams represented in final volume (0.25 g, if a 25.0 mL aliquot was used).

If the nitrite is present as KNO_2 , multiply NaNO_2 content (ppm) by the ratio

$$\frac{\text{KNO}_2}{\text{NaNO}_2} = \frac{85}{69} = 1.23$$

$$\text{KNO}_2 \text{ content (ppm)} = (1.23)(\text{NaNO}_2 \text{ content, ppm})$$

2. Reference

Official Methods of Analysis of the Association of Official Analytical Chemists, 15th Edition, 973.31.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Determination of Nitrite in Meat.								
2. Required Protective Equipment	Safety glasses, plastic gloves, and lab coat.								
3. Procedure Steps	No unusual safety hazards in this method.								
4. Disposal Procedures	<table><tr><th></th><th><u>Hazards</u></th><th><u>Recommended Safe Procedures</u></th></tr><tr><td>Aqueous reaction products</td><td>Mild irritant, pollutant</td><td>Flush down disposal sink with large amounts of water.</td></tr></table>				<u>Hazards</u>	<u>Recommended Safe Procedures</u>	Aqueous reaction products	Mild irritant, pollutant	Flush down disposal sink with large amounts of water.
	<u>Hazards</u>	<u>Recommended Safe Procedures</u>							
Aqueous reaction products	Mild irritant, pollutant	Flush down disposal sink with large amounts of water.							

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standard	Compound	Analytical Range (ppm)	Repeatability % CV	Reproducibility % CV
	Nitrites in Meat	‡	(± 5 ppm)	Not determined
‡ Limit may vary due to sample and aliquot sizes and sample type.				
2. Critical Control Points and Specifications	Record		Acceptable Control	
	Sulfanilamide solution		Prepare fresh daily. Store in brown glass bottle.	
	N-(1-naphthyl) ethylenediamine dihydrochloride solution		Store in brown bottle. Discard if brownish color develops.	
	Filter paper		Check each box for nitrite contamination. Discard if contaminated.	
	Sample weight		5.00 g ± 0.02 g	
	Sample solution pH		6.5-7.0	
	Time interval (step F. 1. d)		>5 minutes	
	Time interval (step F. 1. e.)		Absorbance must be read within 15-30 minutes after addition of NED.	
	Spectrophotometry		Zero instrument with reagent blank.	
	Standard curve		Weight of <i>dried</i> sodium nitrite (2 hrs at 100° C ± 2° C), 0.2000 g ± 0.0002. Each analyst must prepare his/her own curve. At least a single point must be checked with each day's samples. The absorbance of that one point must agree within 5% of the absorbance obtained on the original curve; for example, 0.200 ± 0.010 absorbance units. A new curve must be prepared for each spectrophotometer used. (Curves may be prepared to cover level of interest.) Linear regression equations may be used.	
	Calculations		Recheck.	

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

3. Readiness To Perform

-
- a. Familiarization.
 - i. Phase I: Standards—Prepare standard curve.
 - ii. Phase II: Fortified samples—Random replicates selected by supervisor.
 - b. Acceptability criteria.

See section J.1 above.
-

4. Intralaboratory Check Samples

-
- a. System, minimum contents.
 - i. Frequency: Not to exceed 20% of sampling.
 - ii. Random replicates chosen by supervisor after initial analysis.
 - iii. Records are to be maintained by analyst and reviewed by the supervisor and Laboratory QA Officer.
 - b. Acceptability criteria.

If unacceptable values are obtained, then:

 - i. Stop all official analyses for that analyst.
 - ii. Investigate and identify probable cause.
 - iii. Take corrective action.
 - iv. Repeat Phase II of section J.3 if cause was analyst-related.
-

5. Sample Acceptability and Stability

-
- a. Matrix: Cured meat products.
 - b. Sample receipt size, minimum: 500 g.
 - c. Condition upon receipt: Frozen.
 - d. Sample storage:
 - i. Time: 1 week.
 - ii. Condition: -20° C.
-

6. Sensitivity

-
- a. Lowest detectable level (LDL): NA.
 - b. Lowest reliable quantitation (LRQ): Lowest positive level on standard curve.
 - c. Minimum proficiency level (MPL): 5 ppm.
-

NITRITES IN CURES/PICKLES (ASCORBATES)

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DETERMINATIVE METHOD

A. INTRODUCTION

Theory

This method is very similar to NTI1, in which a spectrophotometric analysis is performed following diazotization and coupling. In an acidified solution, ascorbate and nitrite will react with each other to their mutual destruction, ascorbate being oxidized and the nitrite reduced. To minimize the effects of this reaction on nitrite determinations, a procedure is used that requires 5 min for diazotization and 15 min for coupling to obtain better than a 90% recovery of the nitrite present.

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

-
- a. Balance sensitive to 0.1 mg increments.
 - b. 100, 200, and 1000 mL volumetric flasks.
 - c. 5, 10, 20, and 25 mL volumetric pipettes.
 - d. Filter paper (Reeve Angel #802 or equivalent).
-

2. Instrumentation

Spectrophotometer capable of reading cells of 1-5 cm path length.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

- | |
|---|
| a. Sulfanilamide solution: Dissolve 0.5 g in 100 mL of 1:1 HCl (v/v). Store in g-s brown, glass bottle. |
| b. N-(1-naphthyl) ethylenediamine dihydrochloride solution (NED): Dissolve 0.1 g in 100 mL distilled water. |
| c. Barium chloride: 10% (w/v). |
| d. Sodium nitrite for standard solutions. |

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE

1. Determination

-
- a. *Cures*—Weigh 20.0 g of cure into a 200 mL volumetric flask. Add 100 mL of distilled H₂O, shake to dissolve cure, dilute to volume with distilled H₂O, and mix well. Dilute 3.0 mL of this solution to 1 L with distilled H₂O and mix well. If cure contains phosphate, transfer 3.0 mL of the first dilution to a 1 L volumetric flask, add 15 mL BaCl₂ solution, dilute to volume with distilled H₂O, and mix. Filter through a filter paper (Reeve Angel #802 or equivalent).
- i. If the anticipated nitrite content is less than 1%, transfer 10.0 mL of the second solution to a 100 mL volumetric flask.
- ii. If it is between 1 to 10% nitrite, dilute 10.0 mL of the second solution to 100 mL with distilled H₂O; mix well; and transfer appropriate dilution of this third solution to a 100 mL volumetric flask.

For cures containing more than 10% nitrite, make further appropriate dilutions.

- b. *Pickles*—Dilute 3.0 mL of pickle to 1 L with distilled H₂O and mix well. If pickle contains phosphate, transfer 3.0 mL of the pickle to a 1 L volumetric flask; add 15 mL BaCl₂ solution; dilute to volume with distilled H₂O; and mix. Filter through a filter paper (Reeve Angel, #802 or equivalent). Transfer appropriate dilution of this filtrate to a 100 mL volumetric flask.
- c. *Cures and Pickles*—
- i. To appropriate aliquot of cure or pickle solution contained in the final 100 mL volumetric flask, add 2.5 mL of sulfanilamide solution and mix by swirling.
- ii. Allow to stand for 5 min, add 2.5 mL of N-(1-naphthyl) ethylenediamine dihydrochloride solution, dilute to volume with distilled H₂O, and mix well.
- iii. Let stand for 15 min and measure absorbance at 540 nm, setting the spectrophotometer at 0.0 absorbance with a reagent blank. Obtain NaNO₂ concentration from standard curve and calculate NaNO₂ content.
-

DETERMINATIVE METHOD

F. ANALYTICAL QUANTITATION (Continued)

2. Standard Curve

-
- a. Dissolve 0.2000 g dried NaNO_2 (take into account the assay of your NaNO_2 reagent) in distilled H_2O , dilute to 1 L, and mix well.
 - b. Dilute 10.0 mL of this solution to 1 L with distilled H_2O and mix well. Each mL of the final dilution contains $2.0 \mu\text{g}$ NaNO_2 .
 - c. Pipet 0.0, 5.0, 10.0, 20.0, and 25.0 mL of the final dilution into separate 100 mL volumetric flasks; add 2.5 mL of sulfanilamide solution; and mix by swirling.
 - d. Allow to stand for 5 min, add 2.5 mL of N-(1-naphthyl) ethylenediamine dihydrochloride solution, dilute to volume with distilled H_2O , and mix well.
 - e. Let stand for 15 min and measure absorbance at 540 nm, setting the spectrophotometer at 0.0 absorbance with the reagent blank solution prepared above. Plot absorbance vs micrograms NaNO_2 per mL.
-

DETERMINATIVE METHOD

G. CALCULATIONS

1. Procedures (Cures)

$$\text{Percent NaNO}_2 = \frac{(A) (B) (10^{-4})}{C}$$

Where

A = Micrograms NaNO₂ per mL (from standard curve).

B = Final volume to which an aliquot was diluted (100 mL if conducted as written above).

10⁻⁴ = Factor to convert μg/g to percent.

C = Sample weight in grams represented in final volume.

2. Procedure (Pickles)

$$\text{NaNO}_2 \text{ content (lb/100 gal)} = \frac{(A) (B) (10^{-6}) (834.5)}{V_p}$$

Where

A = Micrograms NaNO₂ per mL (from standard curve).

B = Final volume to which an aliquot was diluted (100 mL if conducted as written above).

10⁻⁶ = Factor to convert μg to g.

834.5 = Factor to convert g/mL to lbs/100 gal.

In order to convert g/mL to lbs/100 gal, it is only necessary to multiply the former by the following factors:

$$\frac{\text{g}}{\text{mL}} \times \frac{\text{lbs}}{\text{g}} \times \frac{\text{mL}}{100 \text{ gal}}$$

which becomes

$$\frac{\text{g}}{\text{mL}} \times \frac{\text{lbs}}{453.6 \text{ g}} \times \frac{378,531 \text{ mL}}{100 \text{ gal}}$$

or

$$\frac{\text{g}}{\text{mL}} \times \frac{834.5 \text{ mL Lbs}}{(\text{g}) \cdot (100 \text{ gal})} = \frac{\text{lbs}}{100 \text{ gal}}$$

or

$$\text{NaNO}_2 \text{ content (lbs/100 gal)} = \frac{\text{g NaNO}_2}{\text{mL}} \times \frac{834.5 \text{ mL lbs}}{(\text{g}) \cdot (100 \text{ gal})}$$

V_p = Volume of pickle represented in final volume.

If the nitrite is present as KNO₂, multiply NaNO₂ content (lb/100 gal) by the

$$\text{ratio } \frac{\text{KNO}_2}{\text{NaNO}_2} = \frac{85}{69} = 1.23$$

$$\text{KNO}_2 \text{ content (lb/100 gal)} = (1.23)(\text{NaNO}_2 \text{ content, lb/100 gal})$$

3. Reference

Official Methods of Analysis of the Association of Official Analytical Chemists, 15th Edition, 973.31.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Determination of nitrite in cures and pickles in the presence of ascorbates.		
2. Required Protective Equipment	Safety glasses, plastic gloves, and lab coat.		
3. Procedure Steps	No unusual safety hazards in this method.		
4. Disposal Procedure			
	<u>Hazards</u>		<u>Recommended Safe Procedures</u>
	Aqueous reaction products	Mild irritant, pollutant	Flush down disposal sink with large amounts of water.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standard	Compound	Analytical Range (% or lb/100 gal)	Repeatability % CV	Reproducibility % CV
	Nitrites in cures and pickles (with ascorbates)	‡	<6.0	<8
‡ Limit may vary due to sample and aliquot sizes and sample type.				
2. Critical Control Points and Specifications	Record		Acceptable Control	
	Barium chloride addition		Barium chloride must be added to all samples containing phosphates.	
NOTE: Refer to NTI1 method for sample control points.				
3. Readiness To Perform	a. Familiarization.			
	i. Phase I: Standards—Prepare standard curve.			
	ii. Phase II: Random replicates.			
	b. Acceptability criteria.			
	See section J.1 above.			
4. Intralaboratory Check Samples	a. System, minimum contents.			
	i. Frequency: Not to exceed 20% of samples.			
	ii. Random replicates chosen by supervisor after initial analysis.			
	iii. Records are to be maintained by analyst and reviewed by the supervisor and Laboratory QA Officer.			
	b. Acceptability criteria.			
	If unacceptable values are obtained then:			
	i. Stop all official analyses for that analyst.			
	ii. Investigate and identify probable cause.			
	iii. Take corrective action.			
	iv. Repeat Phase II of section J.3 if cause was analyst-related.			

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

5. Sample Acceptability and Stability

- a. Matrix: Curing mixtures.
- b. Sample receipt size, minimum: 100 g or 100 mL.
- c. Condition upon receipt: Pickles must be cold (4° C).
- d. Sample storage:
 - i. Time: 1 week.
 - ii. Condition: Frozen.

NOTE: Dry mixes may be stored indefinitely at ambient temperature (25° C).

6. Sensitivity

- a. Lowest detectable level (LDL): 0.05%.
 - b. Lowest reliable quantitation (LRQ): 0.05%.
 - c. Minimum proficiency level (MPL): 0.05%.
-



NITRITES IN CURES/PICKLES (NO ASCORBATES)

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DETERMINATIVE METHOD

A. INTRODUCTION

Theory

In this procedure, nitrite is determined by reacting it with potassium iodide in acid medium. In acid medium, nitrite will oxidize iodide quantitatively to iodine, according to the following equation:



The liberated iodine is then titrated with sodium thiosulfate. During this reaction, nitrite is reduced to nitric oxide, which will form higher oxides of nitrogen by reacting with the oxygen of the air; these higher oxides will then react with iodide to form more iodine and nitric oxide, the cycle repeating itself indefinitely, resulting in failure to attain a permanent endpoint. This induced air oxidation of iodide can be avoided by conducting the analysis in an atmosphere of carbon dioxide or any other inert gas.

DETERMINATIVE METHOD

B. EQUIPMENT

Apparatus

-
- a. Analytical balance.
 - b. 200 mL volumetric flask.
 - c. 300 mL Erlenmeyer flask.
 - d. 100 mL graduated cylinder.
 - e. 10 mL and 25 mL volumetric pipets.
 - f. Buret.
-

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

**Reagent and
Solution List**

-
- a. Dilute sulfuric acid—1 volume of concentrated reagent grade H_2SO_4 + 9 volumes of distilled H_2O .
 - b. Potassium iodide: 10% (w/v).
 - c. Carbon dioxide: Cylinder.
 - d. Starch indicator solution: Triturate 2 g of soluble starch and 10 mg HgI_2 with a small amount of distilled H_2O . Add the suspension slowly to 500 mL boiling distilled H_2O , and boil until clear.
 - e. Sodium thiosulfate solution: 0.05N—Dissolve 12.5 g of $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ in recently boiled, cooled, distilled H_2O ; add 0.1 g Na_2CO_3 and dilute to 1 L. Standardize against $\text{K}_2\text{Cr}_2\text{O}_7$.
-

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE

Determination

-
- a. Weigh 20.0 g of cure into a 200 mL volumetric flask. Add 100 mL of distilled H₂O, shake to dissolve cure, dilute to volume with distilled H₂O, stopper, and mix well.
 - b. To a 300 mL Erlenmeyer flask add 20 mL distilled H₂O, 10 mL of dilute H₂SO₄, and 10 mL of 10% KI solution.
 - c. Bubble CO₂ gently through this solution and throughout the reaction.
 - d. Add a 10.0 mL aliquot of the cure solution (for pickles, add a 25.0 mL sample, making no dilutions) to the flask and titrate with standard thiosulfate solution using starch solution as an indicator.

NOTE: The dilution (20.0 g/200 mL) and aliquot (10.0 mL) indicated above are valid for cures containing less than 40% NaNO₂. For cures with greater than 40% NaNO₂ content, use an appropriately larger dilution and/or a smaller aliquot.

DETERMINATIVE METHOD

G. CALCULATIONS

1. Procedures (Cures)

$$\text{Percent NaNO}_2 = \frac{(V) (N) (6.9)}{S}$$

where

V = Volume of standard sodium thiosulfate solution.

N = Normality of standard sodium thiosulfate solution.

6.9 = Meq. wt. of NaNO₂ (includes factor of 100 for percentage).

S = Sample weight in grams represented by aliquot used.

If nitrite is present as KNO₂, multiply percent NaNO₂ by the ratio:

$$\frac{\text{KNO}_2}{\text{NaNO}_2} = \frac{85}{69} = 1.23$$

$$\text{Percent KNO}_2 = 1.23 (\% \text{ NaNO}_2)$$

2. Procedure (Pickles)

Because the amount of nitrite which may be added to pickles is measured in pounds of nitrite per 100 gal of pickles, the result should bear the same units.

First express the result in grams NaNO₂ per mL of pickle, and proceed from there.

$$\text{g NaNO}_2/\text{mL of pickle} = \frac{(V) (N) (0.069)}{V_p}$$

Where

V = Volume of standard sodium thiosulfate solution.

N = Normality of standard sodium thiosulfate solution.

0.069 = Meq. wt. of NaNO₂.

V_p = Volume of pickle used.

—CALCULATION CONTINUED ON NEXT PAGE—

DETERMINATIVE METHOD

G. CALCULATIONS (Continued)

In order to convert g/mL to lbs/100 gal, it is only necessary to multiply the former by the following factors:

$$\frac{\text{g}}{\text{mL}} \times \frac{\text{lbs}}{\text{g}} \times \frac{\text{mL}}{100 \text{ gal}}$$

which becomes

$$\frac{\text{g}}{\text{mL}} \times \frac{\text{lbs}}{453.6 \text{ g}} \times \frac{378,531 \text{ mL}}{100 \text{ gal}}$$

or

$$\frac{\text{g}}{\text{mL}} \times \frac{834.5 \text{ mL Lbs}}{(\text{g}) \cdot (100 \text{ gal})} = \frac{\text{lbs}}{100 \text{ gal}}$$

or

$$\text{NaNO}_2 \text{ content (lbs/100 gal)} = \frac{\text{g NaNO}_2}{\text{mL}} \times \frac{834.5 \text{ mL lbs}}{(\text{g}) \cdot (100 \text{ gal})}$$

If the nitrite is present as KNO₂, multiply NaNO₂ content (lb/100 gal) by the

$$\text{ratio } \frac{\text{KNO}_2}{\text{NaNO}_2} = \frac{85}{69} = 1.23$$

$$\text{KNO}_2 \text{ content (lb/100 gal)} = (1.23)(\text{NaNO}_2 \text{ content, lb/100 gal})$$

3. Reference

Official Methods of Analysis of the Association of Official Analytical Chemists, 15th Edition, 964.13.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Determination of nitrite in cures and pickles in the absence of ascorbates.		
2. Required Protective Equipment	Safety glasses, plastic gloves, and lab coat.		
3. Procedure Steps		<u>Hazards</u>	<u>Recommended Safer Procedures</u>
	C. Reagents		
	(a) Dilute sulfuric acid (d) HgI_2	Can cause skin and eye irritation; highly toxic and can be absorbed through the skin.	Use protective equipment.
4. Disposal Procedure	Aqueous reaction products	Mild irritant, pollutant	Flush down disposal sink with large amounts of water.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standard	Compound	Analytical Range (% or lb/100 gal)	Repeatability % CV	Reproducibility % CV
	Nitrites in cures and pickles (no ascorbates)	‡	<6.0	<8
‡ Limit may vary due to sample and aliquot sizes and sample type.				
2. Critical Control Points and Specifications	Record		Acceptable Control	
	Sodium thiosulfate solution		Use freshly boiled and cooled distilled water.	
	Sample weight		20.0 g ± 0.1 g for cures. 25 mL (pipet) for pickles.	
	Calculations		Recheck.	
3. Readiness To Perform	a. Familiarization. i. Phase I: Standards—Titrate standard curve. ii. Phase II: Random replicates b. Acceptability criteria. See section J.1 above.			
4. Intralaboratory Check Samples	a. System, minimum contents. i. Frequency: Not to exceed 20% of sampling. ii. Random replicates chosen by supervisor after initial analysis. iii. Records are to be maintained by analyst and reviewed by the supervisor and Laboratory QA Officer. b. Acceptability criteria. If unacceptable values are obtained, then: i. Stop all official analyses for that analyst. ii. Investigate and identify probable cause. iii. Take corrective action. iv. Repeat Phase II of section J.3 if cause was analyst-related.			

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

5. Sample Acceptability and Stability

-
- a. Matrix: Curing mixtures.
 - b. Sample receipt size, minimum: 100 mL or 100 g.
 - c. Condition upon receipt: Pickles must be cold (4° C).
 - d. Sample storage:
 - i. Time: 1 week.
 - ii. Condition: Frozen.
-

6. Sensitivity

-
- a. Lowest detectable level (LDL): 0.01%.
 - b. Lowest reliable quantitation (LRQ): 0.01%.
 - c. Minimum proficiency level (MPL): 0.01%.
-

pH OF MEAT

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I. [Reserved]	
J. [Reserved]	

DETERMINATIVE METHOD

B. EQUIPMENT

Apparatus

-
- a. Beaker, 100 mL.
 - b. Fluted filter paper.
 - c. Glass stirring rod.
 - d. pH meter, suitable for reading pH from 0 to 14 in 0.1 increments.
-

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

**Reagent and
Solution List**

-
- a. Distilled water.
 - b. Certified buffer solutions (commercially available or prepare as in Official Methods of Analysis of the Association of Official Analytical Chemists, 15th Edition, 981.12).
-

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE

Determination

-
- a. With a glass stirring rod, slurry 25-50 g of ground sample in a 100 mL beaker with an equal weight of distilled water.
 - b. Force a fluted filter paper part way down into the slurry and let sit 5 min.
 - c. Calibrate the pH meter according to manufacturer's instructions, using item b, section C, Reagent and Solution List, at the pH closest to that expected in the sample.
 - d. Immerse pH electrodes into the filtered solution inside the fluted filter paper.
 - e. Record pH to the nearest 0.1.
-

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Determination of pH of meat.
2. Required Protective Equipment	Safety glasses, plastic gloves, and lab coat.
3. Procedure Steps	No unusual safety hazards in this method.
4. Disposal Procedures	Use good hygienic practice in disposing of the meat slurry.

PHOSPHATE

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DETERMINATIVE METHOD

A. INTRODUCTION

Theory

In this procedure, a partially dried sample is ashed, the phosphates then hydrolyzed to the ortho form, and separated as quinolinium phosphomolybdate.

This method seems very similar to the ammonium phosphomolybdate procedure, but is actually quite different.

Phosphomolybdic acid is formed first (in the presence of citrate), which then forms quinolinium phosphomolybdate (QPM) with the base, quinoline. The citrate in the reagent complexes any ammonium ion, thereby preventing the precipitation of ammonium phosphomolybdate.

The original version of this procedure required two separate solutions in order to form the QPM precipitate: a citric-molybdic acid solution and a quinoline solution. The inclusion of acetone permitted these two solutions to be combined, so that a single reagent could be employed as the precipitant. This reagent is known as the quimociac reagent, and derives its name from **QUI**noline, **MO**lybdate, **CI**trate, and **AC**etone constituents of the mixture.

DETERMINATIVE METHOD

B. EQUIPMENT

Apparatus

Apparatus listed may be substituted with an equivalent.

- a. Glass fiber filter paper: 2.4 cm circles.
 - b. Gooch crucible: Coors No. 4.
 - c. Muffle furnace: Fisher #10-549-110C.
 - d. Oven: Fisher #13-258-11C.
 - e. Desiccator: Fisher #08-632
-

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and
Solution List

-
- a. Dilute nitric acid (1 + 4): 1 volume concentrated HNO_3 + 4 volumes H_2O .
 - b. Quimociac reagent: Dissolve 70 g sodium molybdate dihydrate ($\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$) in 150 mL H_2O . Dissolve 60 g citric acid monohydrate in a mixture of 85 mL of concentrated HNO_3 and 150 mL H_2O and cool. Gradually add the molybdate solution to the citric-nitric acid solution while stirring. Dissolve 5 mL synthetic quinoline, with stirring, in a mixture of 35 mL of concentrated HNO_3 and 100 mL H_2O . Gradually add this solution to the molybdic-nitric acid solution, mix well, and let stand for 24 hours. Filter, add 280 mL acetone, dilute to 1 L with H_2O , and mix. Store in either a noncolored polyethylene bottle or a dark brown glass bottle.
 - c. Concentrated HCl .
-

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE

1. Ashing

- a. Weigh accurately about 2.5 g (no more than 780 mg QPM) of sample into an ashing dish and dry for 30 min at 125° C in a forced-draft oven.
- b. Ash at 550° C until white ash is obtained.
- c. Cool, add 25 mL of dilute nitric acid, and heat on steam bath for 30 min.
- d. Filter into a 500 mL beaker. Wash dish and paper with distilled water so that total volume in the beaker is approximately 100 mL.

2. Wet Ashing (Optional)

The following wet ashing procedure may be used as a screening method. Violative samples must be repeated by the procedure described in F.1.

- a. Weigh ca. 2.0 g comminuted sample into a 200 mL Kohlrausch or sugar flask, using filter paper to wrap the sample to prevent the meat from adhering to the neck of the flask.
- b. Add 5 mL of concentrated hydrochloric acid and 30 mL of concentrated nitric acid to the flask. At this point prepare a reagent blank using a filter paper and the acids.
- c. Place flask on a hot plate (in a hood) and digest the sample until approximately 15 mL of solution remains. *Caution! Do not let go to dryness.*
- d. Cool flask in hood; make to volume with distilled water. Use the bottom of the fat layer as the meniscus. Mix thoroughly.
- e. Filter a portion of the solution, ca. 30 mL, through a filter paper and pipette a 25 mL aliquot (i.e., 0.25 g) into a 500 mL beaker. Add 75 mL of distilled H₂O.

3. Determination

- a. Run a reagent blank in parallel, using 25 mL of dilute nitric acid and 75 mL of distilled H₂O.
- b. Add 50 mL of quimociac reagent, cover with a watch glass, and boil for 1 min (do not use an open flame).
- c. Cool to room temperature (swirl 3-4 times during cooling). Transfer the precipitate to the prepared crucible and wash 5 times with 25 mL portion of distilled H₂O. Allow each portion to drain through (use vacuum) before adding the next portion.

NOTE: Preparation of the crucible: Place Gooch crucible containing a glass fiber filter disc in suction apparatus. Center disc and wash with approximately 50 mL H₂O. Dry crucible at 250° C for 30 min in a forced-draft oven, cool in desiccator, and weigh.

- d. Dry crucible and contents for 30 min at 250° C, cool in desiccator, and weigh.

DETERMINATIVE METHOD

G. CALCULATIONS

1. Procedure

$$\text{Phosphorus content} = \frac{[(100)(A - B)(0.014)]}{C} - 0.0106 (\% \text{ meat protein})$$

Where:

A = Weight of sample precipitate

B = Weight of blank precipitate

C = Sample weight

0.014 = Gravimetric factor derived from:

Atomic weight of phosphorus = 30.97

Molecular weight of QPM = 2212.71 = $(C_9H_7N)_3H_3PO_4 \cdot 12MoO_3$

$$\frac{P}{QPM} = 0.014$$

0.0106 = Factor to correct for the natural phosphorous content of meat protein

Phosphate content = (Phosphorus Content) (F)

$$F = \frac{\text{Anhydrous molecular weight of desired phosphate}}{(X) (\text{Atomic weight of phosphorus})}$$

Where X = number of atoms of phosphorus in one molecule of the phosphate

The following table lists phosphates and their corresponding factors.

<u>Sodium Phosphates</u>	<u>Factor (F)</u>	<u>Potassium Phosphates</u>	<u>Factor (F)</u>
Na_2HPO_4	4.58	K_2HPO_4	5.61
$(NaPO_3)_6$	3.29	—	—
$Na_5P_3O_{10}$	3.96	$K_5P_3O_{10}$	4.82
$Na_4P_2O_7$	4.29	$K_4P_2O_7$	5.32
NaH_2PO_4	3.87	KH_2PO_4	4.39
$Na_2H_2P_2O_7$	3.58	—	—

In the event that the sodium phosphate used is not known, use the 3.96 factor to calculate added phosphate.

In the event that the potassium phosphate used is not known, use the 4.82 factor to calculate added phosphate.

In the event that a mixture of phosphates is used, use the factor for the phosphate present that will result in the highest value.

2. Reference

Official Methods of Analysis of the Association of Official Analytical Chemists, 15th Edition, 969.31B.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Phosphate Determination.											
2. Required Protective Equipment	Safety glasses, heat-resistant gloves, plastic gloves, lab coat.											
3. Procedure Steps	<table><tr><th></th><th><u>Hazards</u></th><th><u>Recommended Safe Procedures</u></th></tr><tr><td>Wet ashing (optional)</td><td></td><td></td></tr><tr><td>Add 5.0 mL of concentrated hydrochloric acid, and 30 mL of concentrated nitric acid to the flask—etc.</td><td>Conc. H₂SO₄ and HNO₃ can cause severe skin and respiratory irritation.</td><td>Perform this task in an efficient fume hood and try to avoid any distractions when dispensing these strong acids.</td></tr></table>				<u>Hazards</u>	<u>Recommended Safe Procedures</u>	Wet ashing (optional)			Add 5.0 mL of concentrated hydrochloric acid, and 30 mL of concentrated nitric acid to the flask—etc.	Conc. H ₂ SO ₄ and HNO ₃ can cause severe skin and respiratory irritation.	Perform this task in an efficient fume hood and try to avoid any distractions when dispensing these strong acids.
	<u>Hazards</u>	<u>Recommended Safe Procedures</u>										
Wet ashing (optional)												
Add 5.0 mL of concentrated hydrochloric acid, and 30 mL of concentrated nitric acid to the flask—etc.	Conc. H ₂ SO ₄ and HNO ₃ can cause severe skin and respiratory irritation.	Perform this task in an efficient fume hood and try to avoid any distractions when dispensing these strong acids.										
4. Disposal Procedures	Dilute H ₂ SO ₄ -HNO ₃ solution	Mild irritant.	Flush with large quantities of water into an acid-resistant disposal sink.									

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standard

<i>Compound</i>	<i>Analytical Range (ppb)</i>	<i>Repeatability % CV</i>	<i>Reproducibility % CV</i>
Phosphate	‡	<15.0	<20

‡ Limit may vary due to sample and aliquot sizes and sample type.

2. Critical Control Points and Specifications

<i>Record</i>	<i>Acceptable Control</i>
Quimociac solution	Must be filtered before use.
Sample size	Meat—2.5 g \pm 0.1 g for dry ash; 2.0 g \pm 0.1 g for wet ash. Cures, spices, etc.—smaller aliquots or greater dilutions are required than for meat. Sample size for any sample must not exceed 25 mg P ₂ O ₅ or a quinolium phosphomolybdate (QPM) precipitate weight of about 0.78 g.
Wet ash	Screening method only. Do not allow to go to dryness. Use hood.
Muffle furnace temperature	550° C (Do not exceed 600° C).
Blank	Must be run with each set.
Final oven temperature and time	250° C \pm 2° C for 30 min after oven recovers to 250° C.
Calculation	Recheck. (Use proper factor for specific phosphate, if known; otherwise, 3.96 sodium or 4.82 potassium.)

3. Readiness To Perform

- a. Familiarization.
 - i. Phase I: Standards—NA.
 - ii. Phase II: Fortified samples or replicates of previously analyzed samples.
 - iii. Phase III: Check samples for analyst accreditation.
- b. Acceptability criteria.
See section J.1 above.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

**4. Intralaboratory
Check Samples**

-
- a. System, minimum contents.
 - i. Frequency: Not to exceed 20% of samples.
 - ii. Blind samples or random replicates chosen by supervisor after initial analysis.
 - iii. Records are to be maintained by analyst and reviewed by the supervisor and Laboratory QA Officer.
 - b. Acceptability criteria.

If unacceptable values are obtained, then:

 - i. Stop all official analyses for that analyst.
 - ii. Investigate and identify probable cause.
 - iii. Take corrective action.
 - iv. Repeat Phase III of section J.3 if cause was analyst-related.
-

**5. Sample
Acceptability
and Stability**

-
- a. Matrix: Processed meat products.
 - b. Sample receipt size, minimum: 12 oz.
 - c. Condition upon receipt: Cold and sealed from air.
 - d. Sample storage:
 - i. Time: 1 month.
 - ii. Condition: 4° C.
-

6. Sensitivity

-
- a. Lowest detectable level (LDL): NA.
 - b. Lowest reliable quantitation (LRQ): 0.05% P.
 - c. Minimum proficiency level (MPL): 0.05% P.
-

PHOSPHORUS IN MEAT/AUTOMATED

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DETERMINATIVE METHOD

A. INTRODUCTION

Theory

Phosphate and MO^{+6} react in acid solution to produce 12-molybdophosphoric acid, which is reduced with 1-amino-2-naphtol-4-sulfonic acid to phosphomolybdenum blue. Maximum absorbance at 660 nm is proportional to amount phosphorus present. Method is applicable to 0.05-0.4% phosphorus.

This determination can be run simultaneously with the determination of nitrogen. Therefore, the apparatus and some reagents are common to both methods.

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

-
- a. Pipet: Automatic zeroing, 50 mL (Kontes Glass Co., K-763280), or equivalent.
 - b. Tubing: Fluran 1'-5000, 0.125" id, Acidflex, or Teflon, 0.133" id.
 - c. Pipetting machine: Automatic Model 60453 with Model 70327 valve syringe (BBL, Division of BioQuest).
 - d. Laboratory mill (Straub Co., Croydon, PA 19020), Model 4-E, or equivalent.
 - e. Top-loading balance.
 - f. Volumetric flasks: 1 L, 2 L, and 3 L.
 - g. 1.5 L beakers.
 - h. 200 mL tall-form beaker.
 - i. Teflon-coated stirring bars.
 - j. Watch glass, 60 mm diameter.

2. Instrumentation

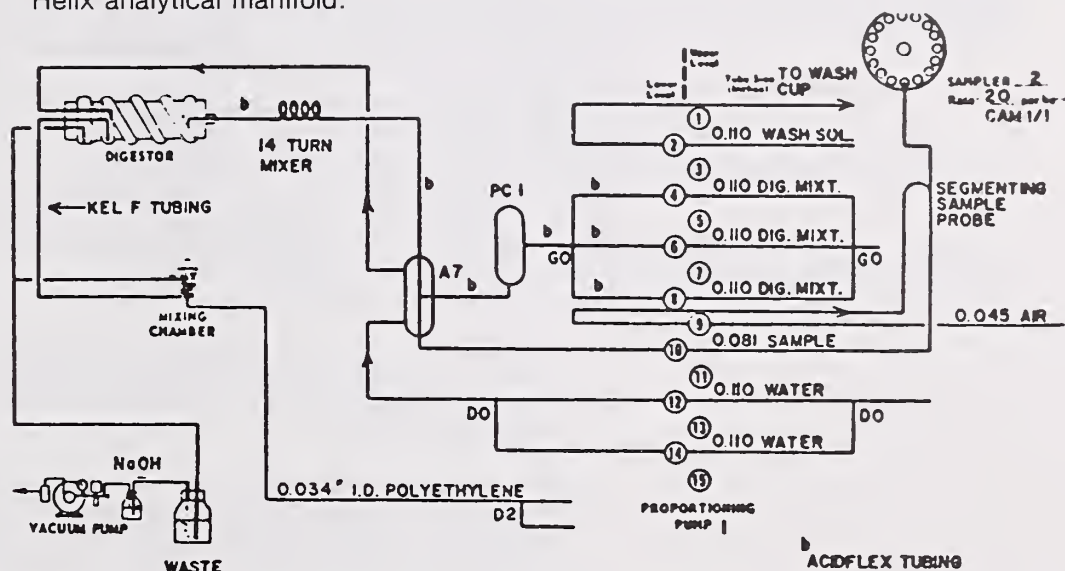
Automatic analyzer. AutoAnalyzer with following modules (Technicon Instruments Corp.): Sampler II; proportioning pump I; continuous digester; proportioning pump II; current stabilizer; constant temperature bath equipped with variable temperature regulator (set at 70° C); colorimeter with 15 mm tubular flowcell, 630 nm filters, and No. 9 aperture; voltage stabilizer; recorder with transmittance paper; vacuum pump; 2 manifolds (Figures 1 and 2, facing page); and 8.5 mL sample cups.

DETERMINATIVE METHOD

B. EQUIPMENT (Continued)

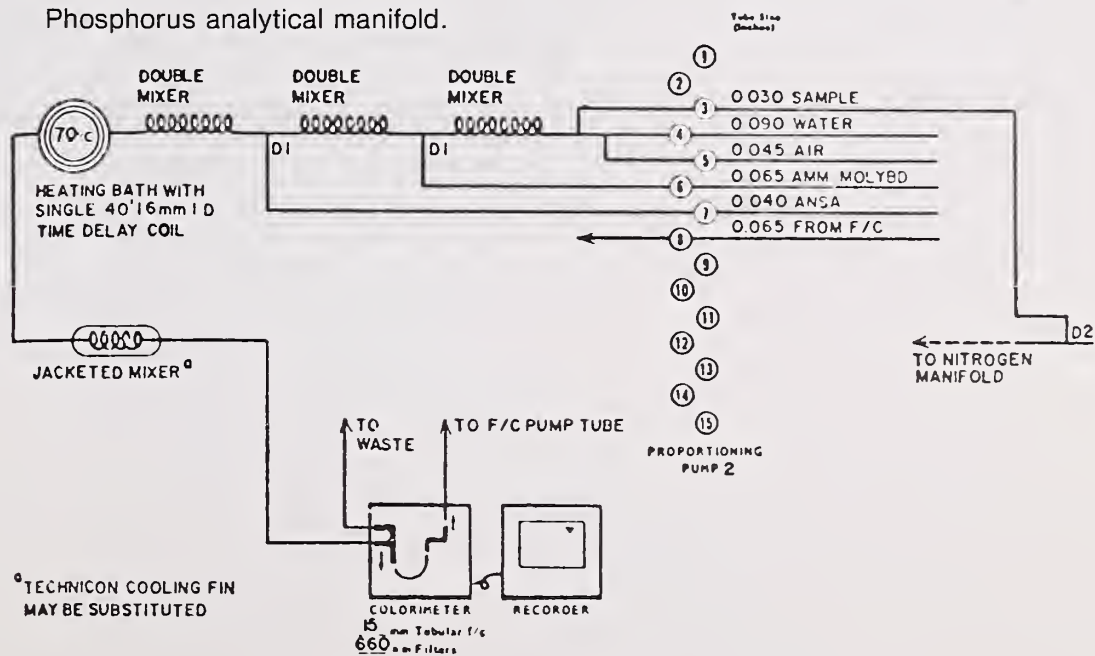
3. Figure 1

Helix analytical manifold.



4. Figure 2

Phosphorus analytical manifold.



DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

**Reagent and
Solution List**

- a. Vanadium pentoxide solution: Weigh 40.0 g NaOH pellets and transfer to 1 L volumetric flask. Add 500 mL H₂O, dissolve, and cool to room temperature. Add 12.5 g V₂O₅ to flask, dissolve, dilute to volume, and mix.
 - b. Digestion mixture: Caution—Mix in order 150 mL V₂O₅ solution, 90 mL 60-62% HClO₄, and 3460 mL H₂SO₄. Rate of consumption is 497 mL/hr.
 - c. Wash solution—H₂SO₄ (1 + 1). Caution: To 1 L H₂O in 2 L volume flask, add 1 L H₂SO₄ slowly with swirling. Cool to room temperature, dilute to volume with H₂O. Rate of consumption is 234 mL/hr.
 - d. 1-amino-2-naphthol-4-sulfonic acid (ANSA).
 - i. Solution A: Add 2.0 g Na₂SO₃ and 60 g NaHSO₃ to 320 mL H₂O in 500 mL volume flask. Heat to 50° C and add 1 g ANSA. Dissolve, cool, dilute to volume, and mix. Store in amber bottle; discard when precipitate forms.
 - ii. Solution B: Dilute 100 mL Solution A to 1 L with H₂O. Add 0.5 mL Levor IV wetting agent (slurry containing 40% sodium nonylbenzene sulfonate, Technicon Instruments Corp.). Store in amber bottle. Refrigerate when not in use. Rate of consumption is 36 mL/hr.
 - e. Ammonium molybdate solution: Dissolve 30 g (NH₄)₆MO₇O₂₄H₂•4H₂O in ca. 1 L H₂O. Dilute to 2 L and mix. Rate of consumption is 96 mL/hr.
 - f. Dilution water.
 - i. Pumped through A7 fitting (Figure 1): Rate of consumption is 468 mL/hr.
 - ii. Pumped through phosphorus analytical manifold (Figure 2): Rate of consumption is 174 mL/hr.
-

DETERMINATIVE METHOD

D. STANDARDS

Preparation of Standard

-
- a. Weigh 10.9839 g KH_2PO_4 into 250 mL flask, add H_2O to dissolve, and dilute to volume.
 - b. Transfer 5.0, 7.5, 10.0, 15.0, 20.0, 30.0, and 40.0 mL to 8 separate 1 L volumetric flasks. Add H_2O to 500 mL.
 - c. Place flasks in ice bath and slowly add 500 mL H_2SO_4 to each. Cool, dilute to volume with H_2O .
 - d. Store in 1 L polyethylene bottles. Based on 10 g sample, percent phosphorus = 0.050, 0.075, 0.100, 0.150, 0.200, 0.250, 0.300, and 0.400.
-

DETERMINATIVE METHOD

E. SAMPLE HANDLING

Sample Preparation

Accurately weigh 10.00 g sample into 200 mL tall-form beaker. Pipet 53 mL H_2O into beaker with pipetting machine. Add 1 " Teflon-coated stirring bar, cover with 60 mm watch glass, and disperse sample, using magnetic stirrer. With stirring, add 50 mL H_2SO_4 using automatic pipet, and continue stirring until sample is dissolved. Cool to room temperature in cooling bath.

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE

1. Analytical System

Use standard 0.0625" id transmission tubing throughout system unless otherwise specified. Pump sample at 2.5 mL/min and segment with air pumped at 0.8 mL/min. Pump digestion mixture at 8.28 mL/min through PCI fitting and add to sample at A7 fitting. Pass sample stream through 14-turn mixing coil into inlet of digester helix. Aspirate diluted sample into bubble chamber and remove aliquot for analysis at rate of 0.23 mL/min. Dilute aliquot with H₂O pumped at 3.90 mL/min and segment with air at 1.20 mL/min. Pass stream through 14-turn mixer and C3 debubbler, and resample at 0.32 mL/min. Add alkaline tartrate solution at 2.90 mL/min and air at 1.60 mL/min. Then pass stream through jacketed mixer, add alkaline phenol solution at 0.80 mL/min, and pass through double mixer. Add NaOCl solution at 0.42 mL/min and pass stream through 14-turn mixer and ½ time-delay coil for color development. Finally pass stream into colorimeter with 630 nm filter and 15 mm tubular flowcell into waste at 2.00 mL/min.

2. Start-Up Procedure

Place all reagent lines, except Acidflex, in water; turn on both proportioning pumps and digester power. Turn on vacuum pump, setting gauge at 12-15 psi. Pump digester mixture and all analytical reagents through their respective lines to determine that system is operating properly. Prior to routine use, optimize digester unit as follows: Using 2.0 mg N/mL standard in duplicate, vary amperage setting according to following table, and record absorbance. Allow 20 min interval after changing setting to stabilize helix temperature before standard is analyzed. Use settings giving highest absorbance.

<u>Amperage Settings</u>		<u>Amperage Settings</u>	
Stage 1	Stages 2 and 3	Stage 1	Stages 2 and 3
2.50	3.00	4.20	6.40
3.00	4.00	4.50	7.00
3.50	5.00	5.00	8.00
3.80	5.60	5.50	9.00
4.00	6.00		

Set digester helix to rotate at 6.7 rpm, referring to Technicon Manual T-69-123 (1970) for instructions.

3. Determination

- Pour standard and prepared samples into 8.5 mL cups and place in Sampler II turntable.
- Adjust sampling rate to 20/hr, with 1:1 sample-to-wash ratio to provide 1.5 min sampling and 1.5 min wash.
- Press reset button and activate sampler turntable, thus passing standards and samples into analytical system. Place stop bar in turntable. (Formation of excessive fat deposits in sample line between segmenting sample probe and input manifold can be retarded by passing wash solution through double mixer wrapped with heating tape and covered with layer of aluminum foil and layer of asbestos; adjust temperature to 60° C with variable transformer connected to heating tape.)

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE (Continued)

-
- d. Read absorbance of samples from recorder strip chart and compare with standard curves of absorbance against % phosphorus on 1-cycle, 70-division semilog paper. A strip chart paper may also be used.
 - e. Include standard curve with every 30 samples. (Percent phosphorus can be converted to percent Na tripolyphosphate, using gravimetric factor 3.96, after percent phosphorus naturally occurring in meat is deducted.) Dilution error caused by variation in moisture content of samples does not significantly affect phosphorus determination. Standard curve is linear through range of standards.
-

4. Shut-Down Procedure

Turn off heat switch and let first stage temperature reach 200° C. Remove helix cover and place all reagent lines except digestion mixture in H₂O after first stage temperature is 150° C. Place digestion mixture line in empty Erlenmeyer and let Acidflex pump tubes "air-wash." Rinse entire system for 15 min. Shut off proportioning pumps and break vacuum in liquid waste bottle. Turn off digester power switch and replace helix cover.

5. Reference

Official Methods of Analysis of the Association of Official Analytical Chemists, 15th Edition 972.22.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Automated Determination of Nitrogen.		
2. Required Protective Equipment	Safety glasses, face shield, heat-resistant gloves, plastic gloves, lab coat, and safety shoes.		
3. Procedure Steps	<u>Reagents</u>	<u>Hazards</u>	<u>Recommended Safe Procedures</u>
	a. Digestion mixture:— ext.	Harmful fumes, corrosive substances, severe skin and respiratory irritation can result.	It is strongly suggested that these reagents be prepared and used in area with exceptional air flow and environmental controls.
	b. Wash solution— H_2SO_4 (1 + 1)—etc.		
	c. Preparation of sample	Harmful fumes and thermal burns from conc. acid.	Protective equipment should be emphasized. The analyst should resist the tendency to <i>rush</i> through this phase, thereby increasing the chances of accidents occurring.
	d. Start-up and shut-down procedures	Ruptured reagent lines and resultant spillage.	Procedures should be followed closely as outlined in the technician manual.
4. Disposal Procedures	Excess meat/acid digestion mixture. Reagent waste	Harmful fumes and thermal burns.	These liquids should be flushed with large quantities of water into an acid-resistant disposal sink. The area should be well ventilated.

PHOSPHATE IN PICKLE

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DETERMINATIVE METHOD

A. INTRODUCTION

Theory

In this procedure, a weighed aliquot is hydrolyzed to convert all phosphates to the ortho form, and separated as quinolinium phosphomolybdate.

This method seems very similar to the ammonium phosphomolybdate procedure, but is actually quite different.

Phosphomolybdic acid is formed first (in the presence of citrate), which then forms quinolinium phosphomolybdate (QPM) with the base, quinoline. The citrate in the reagent complexes any ammonium ion, thereby preventing the precipitation of ammonium phosphomolybdate.

The original version of this procedure required two separate solutions in order to form the QPM precipitate: a citric-molybdic acid solution and a quinoline solution. The inclusion of acetone permitted these two solutions to be combined, so that a single reagent could be employed as the precipitant. This reagent is known as the quimociac reagent, and derives its name from **QU**inoline, **MO**lybdate, **CI**trate, and **AC**etone constituents of the mixture.

DETERMINATIVE METHOD

B. EQUIPMENT

Apparatus

-
- a. Glass fiber filter paper: 2.4 cm circles.
 - b. 200 mL Kohlrausch or sugar flask.
 - c. 500 mL beaker and watch glass.
 - d. Hood.
 - e. Hot plate.
 - f. Gooch crucible: Coors No. 4.

Preparation of the crucible: Place Gooch crucible containing a glass fiber filter disk in suction apparatus. Center the disk and wash with approximately 50 mL H_2O . Dry crucible at 250° C for 30 min in a forced-draft oven, cool in desiccator, and weigh.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

-
- a. Concentrated HCl.
 - b. Concentrated HNO_3 .
 - c. Quimociac reagent: Dissolve 70 g sodium molybdate dihydrate ($\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$) in 150 mL H_2O . Dissolve 60 g citric acid monohydrate in a mixture of 85 mL of concentrated HNO_3 and 150 mL H_2O and cool. Gradually add the molybdate solution to the citric-nitric acid solution while stirring. Dissolve 5 mL synthetic quinoline, with stirring, in a mixture of 35 mL of concentrated HNO_3 and 100 mL H_2O . Gradually add this solution to the molybdic-nitric acid solution, mix well, and let stand for 24 hr. Filter, add 280 mL acetone, dilute to 1 L with H_2O , and mix. Store in either a noncolored polyethylene bottle or a dark brown glass bottle.
-

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE

Determination

-
- a. Pipet 2 mL pickle solution into a 200 mL preweighed Kohlrausch or sugar flask. Obtain the weight of the sample by difference.
 - b. Add 5 mL concentrated HCl and 30 mL concentrated HNO₃. At this point, run a reagent blank in parallel, using the same amounts of these two acids.
 - c. In a hood, place flask on hot plate and digest the sample until approximately 15 mL of solution remains.
 - d. Cool flask in hood; make to volume with distilled water. Mix thoroughly.
 - e. Filter through filter paper into a 300 mL beaker. Pipette 25 mL of solution into a 500 mL beaker. Add 75 mL of distilled H₂O.
 - f. Add 50 mL of filtered quimociac reagent, cover with watch glass, and boil for 1 min. (Do not use an open flame.)
 - g. Cool to room temperature while swirling carefully; transfer the precipitate to the prepared crucible and wash five times with 25 mL portions of distilled H₂O, allowing each portion to drain thoroughly (use suction) before adding the next portion.
 - h. Dry crucible and contents for 30 min at 250° C, cool in desiccator, and weigh.
-

DETERMINATIVE METHOD

G. CALCULATIONS

1. Procedure

$$\text{Phosphorus content} = \frac{[(100)(A - B)(0.014)]}{C}$$

Where:

A = Weight of sample precipitate

B = Weight of blank precipitate

C = Sample weight

0.014 = Gravimetric factor derived from:

Atomic weight of phosphorus = 30.97

Molecular weight of QPM = 2212.7 = $(C_9H_7N)_3H_3PO_4 \cdot 12MoO_3$

$$\frac{P}{QPM} = 0.014$$

Phosphate content = (Phosphorus Content) (F)

$$F = \frac{\text{anhydrous molecular weight of desired phosphate}}{(X) (\text{Atomic weight of phosphorus})}$$

Where X = number of atoms of phosphorus in one molecule of the phosphate

The following table lists phosphates and their corresponding factors.

<u>Sodium Phosphates</u>	<u>Factor (F)</u>	<u>Potassium Phosphates</u>	<u>Factor (F)</u>
Na ₂ HPO ₄	4.58	K ₂ HPO ₄	5.61
(NaPO ₃) ₆	3.29	—	—
Na ₅ P ₃ O ₁₀	3.96	K ₅ P ₃ O ₁₀	4.82
Na ₄ P ₂ O ₇	4.29	K ₄ P ₂ O ₇	5.32
NaH ₂ PO ₄	3.87	KH ₂ PO ₄	4.39
Na ₂ H ₂ P ₂ O ₇	3.58	—	—

In the event that the sodium phosphate used is not known, use the 3.96 factor to calculate added phosphate.

In the event that the potassium phosphate used is not known, use the 4.82 factor to calculate added phosphate.

In the event that a mixture of phosphates is used, use the factor for the phosphate present that will result in the highest value.

2. Reference

Official Methods of Analysis of the Association of Official Analytical Chemists, 15th Edition.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Phosphate Determination in Pickle Solutions.		
2. Required Protective Equipment	Safety glasses, heat-resistant gloves, plastic gloves, lab coat.		
3. Procedure Steps		<u>Hazards</u>	<u>Recommended Safe Procedures</u>
	Wet ashing (optional)		
	Add 5.0 mL of concentrated hydrochloric acid, and 30 mL of concentrated nitric acid to the flask—etc.	Conc. H_2SO_4 and HNO_3 can cause severe skin and respiratory irritation.	Perform this task in an efficient fume hood and try to avoid any distractions when dispensing these strong acids.
4. Disposal Procedures	Dilute H_2SO_4 - HNO_3 solution	Mild irritant.	Flush with large quantities of water into an acid-resistant disposal sink.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standard

<i>Compound</i>	<i>% Phosphorous</i>	<i>Repeatability % CV</i>	<i>Reproducibility % CV</i>
Phosphate	‡	<15.0	<20

‡ Limit may vary due to sample and aliquot sizes and sample type.

2. Critical Control Points and Specifications

<i>Record</i>	<i>Acceptable Control</i>
Quimociac solution	Must be filtered before use.
Sample size	2 mL pickle solution.
Blank	Must be run with each set.
Final oven temperature and time	250° C \pm 2° C for 30 min after oven recovers to 250° C.
Calculation	Recheck. (Use proper factor for specific phosphate, if known; otherwise, 3.96 sodium or 4.82 potassium.)

3. Readiness To Perform

- a. Familiarization.
 - i. Phase I: Standards—NA.
 - ii. Phase II: Fortified samples or replicates of previously analyzed samples.
 - iii. Phase III: Check samples for analyst accreditation.
- b. Acceptability criteria.

See section J.1 above.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

4. Intralaboratory Check Samples

-
- a. System, minimum contents.
 - i. Frequency: Not to exceed 20% of samples.
 - ii. Blind samples or random replicates chosen by supervisor after initial analysis.
 - iii. Records are to be maintained by analyst and reviewed by the supervisor and Laboratory QA Officer.
 - b. Acceptability criteria.

If unacceptable values are obtained, then:

 - i. Stop all official analyses for that analyst.
 - ii. Investigate and identify probable cause.
 - iii. Take corrective action.
 - iv. Repeat Phase III of section J.3 if cause was analyst-related.
-

5. Sample Acceptability and Stability

-
- a. Matrix: Pickle solution.
 - b. Sample receipt size, minimum: 50 mL.
 - c. Condition upon receipt: Cold and sealed from air.
 - d. Sample storage:
 - i. Time: 1 month.
 - ii. Condition: 4° C.
-

6. Sensitivity

-
- a. Lowest detectable level (LDL): NA.
 - b. Lowest reliable quantitation (LRQ): 0.05% P.
 - c. Minimum proficiency level (MPL): 0.05% P.
-

PROTEIN

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DETERMINATIVE METHOD

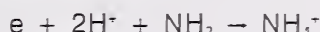
A. INTRODUCTION

1. Theory

Total protein content is determined by the Kjeldahl procedure, in which nitrogen is converted to ammonium bisulfate during digestion, and ammonia liberated therefrom by sodium hydroxide. The ammonia is collected in an excess of standard acid solution, and the excess acid titrated with standard alkali solution.

Approximately 2 g of meat, concentrated sulfuric acid, HgO and K₂SO₄ are added to the Kjeldahl flask. When this mixture is boiled, the protein hydrolyzes to gelatin and finally to amino acids, the basic structural units of proteins and the end result when the protein is completely hydrolyzed.

A solution of NH₄HSO₄, HgO, and KHSO₄ in concentrated sulfuric acid results from the digestion. An essential amino acid, threonine, has the formula CH₃ • CHOH • CHNH₂ • COOH. In order to convert the NH₂ group to NH₄⁺, reducing conditions must exist, as shown by the following partial equation:



The hot acid causes charring and the carbon thus formed is the reducing agent, which acts upon the amino groups, converting or reducing them to NH₄HSO₄.

The HgO is a catalyst, speeding up the reaction considerably. K₂SO₄ is added to raise the boiling point.

Following cooling and dilution of the digest NaOH, Na₂S₂O₃, and zinc or pumice are added and the NH₃ is distilled into a receiver containing an excess of standard acid. The flask should not be shaken until connected to the distillation assembly to prevent loss of NH₃.

The NaOH will release NH₃ from NH₄HSO₄ by double displacement, and the Na₂S is employed to precipitate HgS (black, brown, orange, or yellow depending upon the NaOH concentration). If it were not precipitated, the Hg⁺⁺ would tie up the NH₃ as a Hg amido complex, and low results would be obtained.

Zinc acts as an anti-bumping agent by slowly reacting with the NaOH and forming nascent hydrogen. It is this fine stream of hydrogen that prevents super-heating, the main cause of bumping. Use of Zn, however, probably results in distillation of metallic Hg (nascent H is an excellent reductant). To avoid this, commercial preparations containing HgO, K₂SO₄, and pumice are available and may be used.

The distillate must be on the acid side of the indicator, which may be methyl red, methyl purple, or bromphenol blue.

2. Applicability

This procedure is applicable to the determination of protein content for processed products and animal tissues.

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

-
- a. Analytical balance.
 - b. Erlenmeyer flask: 500 mL.
 - c. Kjeldahl flask: 800 mL.
 - d. Filter paper: 7 cm Whatman #541 or equivalent.
 - e. Rubber stopper: #7.
 - f. Burette: 50 mL.
-

2. Instrumentation

Kjeldahl digestion/distillation equipment.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List	<hr/>
--------------------------------------	-------

- | | | |
|--------------------------------------|-------|---|
| Reagent and
Solution List | <hr/> | <ul style="list-style-type: none">a. Kjeldahl catalyst: 15 g K_2SO_4 + 0.7 g HgO (Commercially prepared catalysts are available containing pumice, if desired).b. Sulfuric acid: A.C.S.c. NaOH solution: Prepare 1200 mL of (1 + 1) NaOH. Allow to stand until clear (ca. 10 days).d. Metallic zinc: Powder, A.C.S., to be used if catalyst does not contain pumice.e. Indicator solution: Fleisher methyl purple or equivalent.f. Acid potassium phthalate: N.B.S. standard.g. Sodium hydroxide: Sodium thiosulfate solution. |
|--------------------------------------|-------|---|

Dissolve 460 g of $Na_2S_2O_3 \cdot 5H_2O$ in water; dilute to 1 L with water; and add this solution to 15,250 g of NaOH dissolved in 14,250 mL of water. This will yield 20 L of a 50 percent (w/w) NaOH solution. If other volumes are desired, adjust weights of NaOH and $Na_2S_2O_3 \cdot 5H_2O$ accordingly. The specific gravity of the final solution should be at least 1.45.

DETERMINATIVE METHOD

D. STANDARDS

Solutions

-
- a. Standard NaOH solution: $0.2000 \pm 0.0004N$

Add 108 mL of (1 + 1) NaOH to CO₂-free distilled H₂O and dilute to 10 L. Standardize against potassium acid phthalate, using phenolphthalein indicator.

- b. Standard acid solution: $0.2000 \pm 0.0004N$. Prepare either hydrochloric or sulfuric acid solution.

(1) Hydrochloric acid: Dilute 178 mL of 35-37 percent reagent grade HCl to 10 L. Standardize against standard NaOH solution and adjust strength accordingly.

(2) Sulfuric acid: Dilute 55 mL of 98 percent reagent grade H₂SO₄ to 10 L. Standardize against standard NaOH solution and adjust strength accordingly.

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE

Determination

-
- a. Accurately weigh, by difference, ca. 2 g of sample on a circle of nitrogen-free filter paper, fold, and transfer to an 800 mL Kjeldahl flask. Run a blank with 1 g sucrose along with and the same way as samples.
 - b. Add catalyst and 40 mL of concentrated H_2SO_4 .
 - c. Digest on Kjeldahl apparatus until solution is clear, and then for at least 30 min longer. When digestion is complete, volume of acid solution in flask should just cover the area of heating element exposed by the porcelain refractory.
 - d. Allow flask and contents to cool to room temperature; carefully add 400 mL of cold tap water, and mix by swirling. Again allow flask and contents to cool to room temperature. If catalyst did not contain pumice, add a pinch of zinc powder.
 - e. To a 500 mL Erlenmeyer flask, add 25.0 mL of 0.2000N HCl or H_2SO_4 , methyl purple indicator, and sufficient distilled H_2O so the end of the delivery tube is submerged. Place the Erlenmeyer flask in position and turn on the heater and the condenser water.
 - f. Add 90 mL of $\text{NaOH}\cdot\text{Na}_2\text{S}_2\text{O}_3$ solution down the side of the Kjeldahl flask, so that it layers on the bottom. Connect the flask to the distilling bulb on the condenser carefully; swirl flask to mix contents thoroughly and place flask on hot heater.
 - g. Collect 150-200 mL of distillate and titrate the excess standard acid with 0.2000N NaOH.
-

DETERMINATIVE METHOD

G. CALCULATIONS

1. Procedure

a. Percent Protein =
$$\frac{(V_a - V_b)(1.4007)(6.25)(N)}{\text{Sample Weight}}$$

Where

V_a = Volume of standard acid added

V_b = Volume of standard base used in titration

1.4007 = Meq. wt of nitrogen (includes factor of 100 for percentage).

6.25 = Factor for converting from nitrogen to protein because protein is 16 percent nitrogen.

N = Normality of standard acid and base.

If both the acid and base are 0.2000N, the formula reduces to:

$$\text{Percent Protein} = \frac{(V_a - V_b)(1.75)}{\text{Sample Weight}}$$

(1) The last equation indicates the importance of being sure that the normalities of the standard solutions are as close to 0.2000N as is practical; 0.1996 to 0.2004 is acceptable. The factor 1.75 assumes that standard solutions of 0.2000N strength are being used.

(2) If the 1.75 factor is used to calculate protein content, and the standard solutions are appreciably higher than 0.2000N, the calculated results will be lower than the true protein content of the sample. Conversely, if the standard solutions are appreciably lower than 0.2000N, and the 1.75 factor is used, the calculated results will be higher than the true protein content of the sample.

b. Total protein content: Percent =
$$\frac{[(A - (B + S))1.75]}{C}$$

A = Volume of standard 0.2000N acid added.

B = Volume of standard 0.2000N NaOH used in sample titration.

S = Volume of standard 0.2000N NaOH used in sucrose blank titration.

C = Sample weight.

2. References

Official Methods of Analysis of the Association of Official Analytical Chemists, 15th Edition.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Protein Determination.		
2. Required Protective Equipment	Safety glasses, face shield, heat-resistant gloves, plastic gloves, and lab coat.		
3. Procedure Steps		<u>Hazards</u>	<u>Recommended Safe Procedures</u>
	F.b. Add catalyst and 40 mL conc. H_2SO_4 .	H_2SO_4 is very corrosive. Severe respiratory irritation can result.	Always check dispensing apparatus in order to prevent spillage or splashing. Do not rush.
	F.d. Allow flask to cool carefully, add 400 mL of cold water to digest, mix, and cool again.	SO_3 vapors are toxic and commonly cause severe respiratory irritation.	Allow flasks to cool before removal from the burners. Add water to the flasks in a fume hood.
	F.f. Add 90 mL NaOH- $Na_2S_2O_3$	NaOH is strongly corrosive and may cause irreversible skin damage.	As in F.b., take precautions to prevent contact with the skin and eyes.
	F.g. Collect 150-200 mL distillate	Mercury is a cumulative toxin, which can produce delirium and hallucinations.	Verify that hood has adequate air flow and that ambient temperature is not greater than 75° F.
4. Disposal Procedures	Precipitated mercury waste is stored and periodically shipped.	Mercury is especially hazardous when in contact with alkali or halogens	Dilute each Kjeldahl reaction mixture to 300 mL with H_2O . Add 50 mL 30% H_2O_2 . Warm gently and allow HgS to ppt completely. Separate and store for disposal.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standard

<i>Compound</i>	<i>Analytical Range (%)</i>	<i>Acceptable Recovery %</i>	<i>Repeatability % CV</i>	<i>Reproducibility % CV</i>
Protein	‡	99.4-100.6	< 0.24†	< 0.32†

‡ Limit may vary due to sample and aliquot sizes and sample type.

† Standard deviation.

2. Critical Control Points and Specifications

<i>Record</i>	<i>Acceptable Control</i>
Kjeldahl flasks	Conduct 6-inch drop test, or equivalent, before using. Use safety precautions.
Kjeldahl units	Water flushed weekly and after each flowback or overflow. Replace old, worn, cracked, or hardened tubing and stoppers. Repair burners after spillage of broken filaments. (Also see ammonium sulfate recoveries, below.)
Catalyst	15.7 g \pm 0.2 g from Pope Dispenser or use "Kel Paks."
Titralyzer	Check for proper working order and calibrate daily. Record in "Standards Book."
Burets	Calibrate at 10 mL intervals. Record in "Standards Book."
Sample size	2 g \pm 0.3 g (except high, >20%, protein samples and low, <8%, protein samples require weight adjustment accordingly).
Volume of concentrated sulfuric acid	40 mL \pm 2 mL.
Digestion time	At least 30 min after clearing.
Flask rotation	At least 2 times during digestion.
Strength of standard acid and base	0.2000N \pm 0.0004N. Record standardizations in "Standards Book." (Other normalities with equivalent quality control and proper calculations may be used.)
Ammonium sulfate, standard protein, or standard amino acid recoveries	99.5% \pm 0.5%. Utilize all burners, keep documentation in Standards Book." (Suggested frequency, 1 per month.)

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

Blanks	25.00 mL \pm 0.10 mL standard base per 25.00 mL standard acid used for calibrated burets, \pm 0.20 mL for titralyzer. Method blank performed with 1 g sucrose.
"Green distillations"	Start over with reduced sample size.
Calculation	Recheck.

3. Readiness To Perform

- a. Familiarization.
 - i. Phase I: Standards—NA.
 - ii. Phase II: Fortified samples.
 - iii. Phase III: Check samples for analyst accreditation.
 - b. Acceptability criteria.
- See section J.1 above.

4. Intralaboratory Check Samples

- a. System, minimum contents.
 - i. Frequency: Not to exceed 20% of samples.
 - ii. Blind samples or random replicates chosen by supervisor after initial analysis.
 - iii. Records are to be maintained by analyst and reviewed by the supervisor and Laboratory QA Officer.
 - b. Acceptability criteria.
- If unacceptable values are obtained, then:
- i. Stop all official analyses for that analyst.
 - ii. Investigate and identify probable cause.
 - iii. Take corrective action.
 - iv. Repeat Phase III of section J.3 if cause was analyst-related.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

**5. Sample
Acceptability
and Stability**

-
- a. Matrix: Meat and meat product.
 - b. Sample receipt size, minimum: 500 g.
 - c. Condition upon receipt: Cool 4° C.
 - d. Sample storage:
 - i. Time: 6 months.
 - ii. Condition: Frozen.
-

6. Sensitivity

-
- a. Lowest detectable level (LDL): ND
 - b. Lowest reliable quantitation (LRQ): 0.5%.
 - c. Minimum proficiency level (MPL): 0.5%.
-

PROTEIN (ALTERNATE)

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DETERMINATIVE METHOD

A. INTRODUCTION

Theory

The Kjeldahl method for protein in meat using boric acid traps—This method involves heating the material for some time with concentrated sulfuric acid at or near its boiling point in the presence of a catalyst, followed by a distillation in alkaline solution, catching the nitrogen converted to ammonia in a boric acid trap, and titrating it directly with standard acid.

Refer also to method PRO1, section A.

DETERMINATIVE METHOD

B. EQUIPMENT

Apparatus

-
- a. Kjeldahl digestion: distillation equipment.
 - b. Erlenmeyer flask: 500 mL.
 - c. Kjeldahl flask: 800 mL.
 - d. Filter paper: 7 cm Whatman #541 or equivalent.
-

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

-
- a. Kjeldahl catalyst: 15 g potassium sulphate + 0.7 g mercuric oxide (Commercially prepared catalysts are available containing pumice, if desired.).
 - b. Sulfuric acid: A.C.S.
 - c. Sodium hydroxide (NaOH) solution: Prepare 1200 mL of (1.1) NaOH. Allow to stand until clear (ca. 10 days).
 - d. Metallic zinc: Powder, A.C.S., to be used if catalyst does not contain pumice.
 - e. Mixed indicator: Mix 2 parts of 0.05% methyl red (in 95% ethanol) with 3 parts 0.075% bromcresol green (in 95% ethanol), or N-Point Indicator (MCB #NX847).
 - f. Potassium hydrogen phthalate: N.B.S. Standard.
 - g. Standard NaOH solution: $0.2000 \pm 0.0004N$.
Add 10.1 mL of (1 + 1) NaOH to CO_2 -free distilled H_2O and dilute to 1 L. Standardize against potassium hydrogen phthalate, using phenolphthalein indicator.
 - h. Standard acid solution: $0.2000 \pm 0.0004N$. Prepare either hydrochloric or sulfuric acid solution.
 - i. Hydrochloric acid: Dilute 178 mL of 35-37% reagent grade HCl to 10 L. Standardize against standard NaOH solution and adjust strength accordingly.
 - j. Sulfuric acid: Dilute 55 mL of 98% reagent grade sulfuric acid to 10 L. Standardize against standard NaOH solution and adjust strength accordingly.
 - k. Sodium hydroxide solution [50% (w/w)]: Dissolve 15,250 g of NaOH in 14,250 mL of water.

NOTE: Commercially available sodium hydroxide solution [50% (w/w)] can be used instead of preparing the reagent in the laboratory.
 - l. Sodium thiosulfate solution: Dissolve 900 g of $Na_2S_2O_3 \cdot 5H_2O$ in water, dilute to 1 L.

NOTE: This reagent does not need to be used if a copper-based catalyst is used in the digestion of the sample.

NOTE: If reagent k is prepared in the laboratory, this solution may be added to it; if so, step f in section F can be skipped.
 - m. Saturated boric acid solution: 60 g H_3BO_3/L : Stir well and let sit over night before use. Filter before use and add 20 mL mixed indicator/L of solution. (See item e above.)
-

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE

Determination

-
- a. Accurately weigh by difference ca. 2 g of sample on a circle of nitrogen-free filter paper, fold, and transfer to an 800 mL Kjeldahl flask. (Run a blank of 1 g sucrose along with and the same way as samples.)
 - b. Add catalyst and 40 mL of concentrated H_2SO_4 .
 - c. Digest on Kjeldahl apparatus until solution is clear, and then for at least 30 min longer. When digestion is complete, volume of acid solution in flask should just cover the area of heating element exposed by the porcelain refractory.
 - d. Allow flask and contents to cool to room temperature; carefully add 400 mL of cold tap water, and mix by swirling. Again allow flask and contents to cool at room temperature. If catalyst did not contain pumice, add a pinch of zinc powder.
 - e. To a 500 mL Erlenmeyer flask, add 50.0 mL of saturated boric acid/mixed indicator solution, and sufficient distilled H_2O so the end of the delivery tube is submerged. Place the Erlenmeyer flask in position and turn on the heater and the condenser water.
 - f. Add 5 mL of the sodium thiosulfate solution to the Kjeldahl flask.

NOTE: This step can be skipped if reagents k and l were combined into one solution.
 - g. Add 90 mL of $\text{NaOH-Na}_2\text{S}_2\text{O}_3$ solution down the side of the Kjeldahl flask, so that it layers on the bottom. Connect the flask to the distilling bulb on the condenser carefully, swirl the flask to mix the contents thoroughly, and place the flask on the hot heater.
 - h. Collect 150-200 mL of distillate and titrate the blank first, with 0.2000N HCl or H_2SO_4 , to a gray-pink end point. Match the sample end point with the blank end point color.
-

DETERMINATIVE METHOD

G. CALCULATIONS

1. Procedure

$$\text{Total protein content: Percent} = \frac{(A - B)(1.4007)(6.25)(N)}{C}$$

A = Volume of standard 0.2000N acid used in sample titration.

B = Volume of standard 0.2000N acid used in blank titration.

C = Sample weight.

1.4007 = Meq. wt of nitrogen (includes factor of 100 for percentage).

6.25 = Factor for converting from nitrogen to protein because protein is 16 percent nitrogen.

N = Normality of standard acid and base.

If both the acid and base are 0.2000N, the formula reduces to:

$$\text{Total protein content: Percent} = \frac{(A - B)(1.75)}{C}$$

2. References

- a. Official Methods of Analysis of the Association of Official Analytical Chemists, 15th Edition.
 - b. Kjeldahl, Z., Anal. Chem. 22:336-82 (1883).
 - c. Wilferth, Chem. Zentr. 56:17, 113 (1885).
 - d. Winkler, Z. Angew. Chem. 26: 231 (1913).
 - e. Winkler, Z. Angew. Chem. 27: 630 (1914).
-

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Alternate Protein Determination.
2. Required Protective Equipment	Safety glasses, face shield, heat-resistant gloves, plastic gloves, lab coat, and safety shoes.
3. Procedure Steps	Refer to method PRO1, section H. This method substitutes boric acid traps but is otherwise identical to method PRO1.

SODIUM, POTASSIUM, CHLORINE (NOVA)

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DETERMINATIVE METHOD

A. INTRODUCTION

Introduction

The NOVA analyzer uses ion specific electrode technology to measure the activity of Na^+ , K^+ , and Cl^- in aqueous solution. The instrument, originally designed to measure blood electrolyte levels in whole blood, has been modified to measure Na^+ , K^+ , and Cl^- in aqueous extracts of food products. The instrument is highly automated and self-contained. Calibration fluids and sample dilution buffers are contained in a fluids pack within the cabinet. Operation is simple, and routine maintenance may be performed by a properly trained operator.

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

-
- a. Macropipette, 50 μ L: Supelco #5-8471 or equivalent.
 - b. Sample cups: NOVA Biomedical Corp., 20 Ossipee Rd., Newton, MA 02164 or equivalent.
 - c. Centrifuge tubes, disposable polypropylene, 50 mL capacity with screw caps: Corning #25330 or equivalent.
 - d. Disposable micropipette tips: Supelco #5-8461 or equivalent.
 - e. Disposable macropipette filter tips (45 micron): Supelco #5-8474 or equivalent.
 - f. Eberbach mechanical shaker or equivalent.
-

2. Instrumentation

NOVA analyzer: NOVA Biomedical Corp., equipped with Na⁺, K⁺, and Cl⁻ electrodes, auto sample unit, and printer.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and Solution List

- | |
|---|
| a. Deionized water: with resistivity greater than 10 megohms. |
| b. Electrode cleaning solution. |

DETERMINATIVE METHOD

D. STANDARDS

1. Source

Fluid pack NOVA Biomedical Corp., 20 Ossipee Rd., Newton, MA 02164.

- a. High standard: Na⁺ (230 ppm), K⁺ (390 ppm), Cl⁻ (709 ppm).
 - b. Low standard: Na⁺ (23 ppm), K⁺ (39 ppm), Cl⁻ (70.9 ppm).
 - c. Sample dilution buffer.
 - d. Reference electrode solution.
-

2. Preparation of Standards

Sodium conditioning solution.

a. Stock solutions.

- i. 1000 μ g/mL Na⁺: Dry reagent grade NaCl 2 hr at 100° C, cool, and weigh 2.5421 g into a 1 L volumetric flask. Dilute to volume with deionized water.
- ii. 1000 μ g/mL K⁺: Dry reagent grade KCl 2 hr at 100° C, cool, and weigh 1.9068 g into a 1 L volumetric flask. Dilute to volume with deionized water.

b. Working solutions.

Prepare working standard solutions by making the following dilutions from the 1000 μ g/mL stock solutions using deionized water.

<i>Vol Na⁺ Stock</i>	<i>Vol K⁺ Stock</i>	<i>Final Volume</i>	<i>Na⁺</i>	<i>(ppm) K⁺</i>	<i>Cl⁻</i>
10 mL	7.5 mL	200 mL	50	37.5	111
20 mL	15 mL	200 mL	100	75.0	222
40 mL	30 mL	200 mL	200	150	444
80 mL	60 mL	200 mL	400	300	888

c. Recovery spiking solution.

Dry reagent grade NaCl and KCl 2 hr at 100° C. Cool and weigh 7.6203 g NaCl and 1.7161 g KCl into a 500 mL volumetric flask. Make to volume with deionized water.

DETERMINATIVE METHOD

E. SAMPLE HANDLING

1. Preparation

Solid or semi-solid sample materials should be homogenized. Fluid or semi-fluid sample materials such as soups, stews, etc., should be blended. A standard commercial-style food processor has been found to be adequate for this purpose. Waring blenders or Virtis homogenizers are also acceptable.

2. Storage

Sub-samples of homogenized material should be stored frozen until analyzed. Plastic bags are recommended for storage.

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE

1. Determination

- a. Instrument calibration. Calibrate the NOVA instrument according to manufacturer's instructions. Internal electrode calibration is performed automatically using standard reference solutions contained within the fluid pack. Do not proceed until a satisfactory calibration is obtained.
- b. Standard curve. Analyze the working standards prepared in section D.2.b. Calculate a calibration curve of the form $y = mx + b$ for each ion where y = actual ppm present, x is ppm determined by NOVA, m is the slope, and b the intercept. Calculate m and b using the following formulas:

$$b = \frac{\Sigma xy - \frac{\Sigma x \Sigma y}{n}}{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}$$

$$m = \bar{y} - b \bar{x}$$

$$\text{where } \bar{y} = \frac{\Sigma y}{n}; \bar{x} = \frac{\Sigma x}{n}$$

$$\text{regression coefficient} = \frac{\left[\Sigma xy - \frac{\Sigma x \Sigma y}{n} \right]^2}{\left[\Sigma x^2 - \frac{(\Sigma x)^2}{n} \right] \left[\Sigma y^2 - \frac{(\Sigma y)^2}{n} \right]}$$

Do not proceed with sample analysis until satisfactory linear regression data are obtained. Each set of samples should contain the following quality assurance samples:

- i. Internal check. A sample that has been analyzed previously and that has a known Na^+ , K^+ , and Cl^- content.
 - ii. Recovery blank. A sample to which no Na^+ , K^+ , and Cl^- has been added.
 - iii. Recovery. A sample to which a known amount of Na^+ , K^+ , or Cl^- has been added. Prepare by adding 2.0 mL of recovery spiking solution to recovery blank from above (equivalent to 12,000 μg Na^+ , 3,600 μg K^+ , and 21,768 μg Cl^- added).
- c. Sample extraction.
- i. Weigh 2.5 g of homogenized sample into a 50 mL polypropylene centrifuge tube.
 - ii. Quantitatively add 40 mL \pm 0.1 mL deionized water to each sample.
 - iii. Cap and place horizontally on shaker.

DETERMINATIVE METHOD

F. ANALYTIC PROCEDURE (Continued)

-
- iv. Shake for 30 min.
 - v. Let stand for 15 min.
 - vi. Transfer about 2 mL of the supernatant fluid to a sample cup using a 45 micron filter tip assembly.
 - vii. Analyze on NOVA analyzer as per instrument instructions.
-

DETERMINATIVE METHOD

G. CALCULATIONS

1. Procedure

Data from the NOVA analyzer are reported in ppm on a solution basis and therefore must be converted to mg/100 g of product. The following steps are required to perform this conversion:

- a. Raw data from the NOVA analyzer are corrected using the linear curves calculated in section F.
- b. mg/100 g sample is calculated using the following formula:

$$\text{mg/100 g} = \frac{R \times V}{W \times 10}$$

where:

R = Recovery corrected ppm NOVA reading

V = Volume of extract (usually 100 mL)

W = Sample weight in grams

10 = Conversion factor

Calculations of linear regression coefficients and sample concentrations are easily accomplished using a suitably programmed calculator or computer.

2. Quality Control Criteria

To ensure the reliability of reported results, the following criteria are monitored:

- a. Sample identity is carefully preserved throughout the process. Working conditions are arranged to reduce the possibility of loss of sample identity.
- b. Reagents and solutions are carefully prepared and recorded. Calculation of reagent or reference standard concentrations are checked by a second analyst.
- c. Instrument performance is carefully monitored. Before sample analysis, the instrument must pass the following tests:
 - i. Internal calibration. The instrument must calibrate successfully with an acceptable slope for all three electrodes.
 - ii. Linear regression curves calculated using working standards must have acceptable slopes and regression coefficients.
 - iii. The value for the internal check sample must be within ± 2.2 standard deviations of the mean value previously determined on a minimum of 14 checks.
 - iv. The recovery values must be within acceptable limits. (Refer to section G.3 for expected recovery values.)
 - v. As a final check for each sample, the ratio of Na⁺ to NaCl is calculated. This ratio should be reasonably close to the 0.39 theoretical value.

DETERMINATIVE METHOD

G. CALCULATIONS (Continued)

3. Recovery Values

<i>Added</i>	<i>Mean Recovery</i>	<i>Std Dev.</i>	<i>CV</i>	<i>n</i>
Na ⁺	100.5	2.65	2.64	14
K ⁺	100.7	2.44	2.42	14
Cl ⁻	100.3	4.22	4.21	14

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Determination of Na^+ , K^+ , and Cl^- by Ion-Specific Electrode Using the NOVA Analyzer.	
2. Protective Equipment	Safety glasses, plastic gloves, and lab coat.	
3. Procedure Steps	<u>Hazards</u>	<u>Recommended Safe Procedures</u>
	No known hazardous chemicals are used in this procedure.	Follow generally recognized safe laboratory practice.
4. Disposal Procedures		No special disposal procedures are required.

SODIUM/POTASSIUM IN MEAT BY ATOMIC EMISSION SPECTROSCOPY

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I. [Reserved]	
J. [Reserved]	

DETERMINATIVE METHOD

A. INTRODUCTION

Theory

Due to expected high levels of Na and K in products of interest, atomic emission rather than atomic absorption is used. The sample is defatted and ashed. Wet ashing is the primary ashing technique, but dry ashing may be necessary for some products. The resultant ash is solubilized, diluted, and aspirated into a flame; its emission is then read and compared to a standard curve for quantitation.

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

-
- a. Food chopper equipped with 5/64" perforated front plate.
 - b. Food processor with rotary blades.
 - c. Steam bath.
 - d. Explosion-proof hotplate.
 - e. Convection oven.
 - f. Muffle furnace.
 - g. Platinum crucibles.
 - h. Centrifuge.
 - i. Vinyl gloves.
 - j. Desiccator.
 - k. Analytical balance.
 - l. Glassware: 50 mL Pyrex beakers, class A volumetric flasks and transfer pipets, and 50 mL plastic Falcon tubes with lids. All glassware must be cleaned with 50% HNO₃ and rinsed with H₂O immediately before use.
-

2. Instrumentation

Atomic emission spectrophotometer: P/E Model 603, or equivalent.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and
Solution List

-
- a. Distilled water, free from Na and K: Either double-distilled or deionized.
Use for all " H_2O " references in this method.
 - b. Petroleum ether: Distilled in glass (Burdick and Jackson, or equivalent).
 - c. Nitric acid: Redistilled. *Use for all " HNO_3 " references in this method.*
-

DETERMINATIVE METHOD

D. STANDARDS

Preparation of Standards

a. Sodium standard solutions.

- i. Na stock solution: 1 mg Na/mL. Dry reagent grade NaCl 2 hr at 110° C; cool in desiccator. Weigh 2.5421 g into 1 L volumetric flask and dilute to volume with H₂O.
- ii. Na working solutions (for flame emissions): 20, 40, 80, 150, 300, and 400 µg Na/mL. Pipet 2, 4, 8, 15, 30, and 40 mL Na stock solution into separate 100 mL volumetric flasks. Add 2 mL HNO₃ and 10 mL K stock solution to each flask and dilute to volume with H₂O. Use a solution of 2 mL HNO₃ diluted to 100 mL with H₂O to zero spectrophotometer. Make a new set of working solutions for each set of samples analyzed.

b. Potassium standard solutions.

- i. K stock solution: 1 mg K/mL. Dry reagent grade KCl 2 hr at 100° C; cool in desiccator. Weigh 1.9068 g into 1 L volumetric flask and dilute to volume with H₂O.
 - ii. K working solutions (for flame emissions): 20, 40, 60, 100, 150, and 180 µg/mL. Pipet 2, 4, 6, 10, 15, and 18 mL K stock solution into separate 100 mL volumetric flasks. Add 2 mL HNO₃ and 30 mL Na stock solution to each flask and dilute to volume with H₂O. Use a solution of 2 mL HNO₃ diluted to 100 mL with H₂O to zero spectrophotometer. Make a new set of working solutions for each set of samples analyzed.
-

DETERMINATIVE METHOD

E. SAMPLE HANDLING

Sample Preparation

Convert the sample to a homogeneous mass containing all of the original juices, free liquids, and chunks so that the small portion taken for analysis is representative of the sample.

- a. Comminuted meat food products—Chop twice using a clean, dry chopper equipped with a plate perforated by 5/64" holes, with mixing between passes through the chopper. Maintain sample identification.
 - b. Meat cuts—Chop three times as described in a above.
 - c. Pizza, spaghetti products, meat and poultry pies, soups, and stews—Homogenize, using a food processor with rotating blades, and weigh immediately into a clean 50 mL beaker before settling begins.
-

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE

1. Wet Ashing

- a. Weigh 1.500 to 2.500 g sample to the nearest 0.001 g into a 50 mL Pyrex beaker. Flatten samples to cover at least one-half of the bottom of the beaker before final weight is taken. Keep sample in a flat wafer (as much as possible) rather than small pieces. This will prevent loss of sample during defatting.
- b. Dry 16 hr at 90° C and cool.
- c.
 - i. Samples with unknown oil/fat content or known high oil/fat content: Add about 10 mL pet ether and warm on steam bath or an explosion-proof hot plate, set at low heat, until oil/fat is extracting. Decant and repeat until sample is defatted. Evaporate remaining petroleum ether. Proceed as in c.ii for samples with low oil content.
 - ii. Samples with low oil content: Add 5 mL HNO₃ to each beaker. Digest on low-temperature hot plate (200-250° F) until sample dissolves. Samples containing products other than meat, such as pizza and spaghetti, react faster and must be started below 200° F. Evaporate to dryness. Repeat this digestion and evaporation two more times with 5 mL HNO₃. *(Stop at this point unless samples can be carried through to completion or losses will occur.)*

Add 2 mL HNO₃ and warm to dissolve.

- d. For flame emission, transfer digest to 100 mL volumetric flask with hot H₂O: Wash down sides of beaker three times with hot H₂O and add washings to flask. Cool and dilute to volume with H₂O. Centrifuge 30 mL aliquot in 50 mL Falcon tubes at 2000 rpm for 10 min.
- e. Prepare two blank solutions by carrying two empty 50 mL beakers or crucibles through the method as described.

The average of the blank Na/K emission units are subtracted from the Na/K analytical sample emission units before calculating the Na/K content of the sample
- f. Analyze a recovery with each set by fortifying samples of known Na/K content with approximately 600 mg Na/100 mg and 180 mg K/100 mg.

2. Dry Ashing

For samples that are difficult to wet-ash.

- a. Weigh appropriate amount of samples into a platinum crucible and dry at 90° C for 16 hr.
- b. Place in a cold muffle furnace and slowly bring up to 525° C. Ash until little or no visible carbon is present.
- c. Cool and dampen ash with 2 mL 50% HNO₃.
- d. Dry on hot plate set at 200° F.
- e. Return to cool muffle furnace and bring to 525° C.

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE (Continued)

3. Emission Spectrophotometry

-
- f. Hold for 1 hr and cool. *(Stop at this point unless samples can be carried through to completion.)*
 - g. Dissolve ash with dilute HNO_3 (20 mL HNO_3 /L H_2O).
 - h. Transfer quantitatively to 100 mL volumetric flask and make to volume with dilute HNO_3 .
-
- a. Follow manufacturer's directions for type of instrument available. Read at 589 nm for Na (for the P/E 603: 295 vis-EM chop, slit 4, 4 sec) and at 767 nm for K (for the P/E 603: 383 vis-EM chop, slit 4, 4 sec). Warm burner 20 min to stabilize flame.
 - b. Read standard curve. The curve can be read several ways.
 - i. If the entire curve is to be used (Na: 20 to 400 $\mu\text{g Na/mL}$, K: 20 to 180 $\mu\text{g K/mL}$), keep the highest standard used between 1.6 and 1.7 emission units. Six standards evenly spaced along the curve are normally used.
 - ii. If only a short portion of the curve is to be used, four standards may be used. Even if a short portion is used, set the 180 $\mu\text{g K/mL}$ and 400 $\mu\text{g Na/mL}$ standards at 1.6 to 1.7 emission units.
 - iii. The sample readings must be between the highest and lowest standard used. Check a standard every 5 to 10 samples and watch for zero drift. Treat the blank solutions and the recoveries the same as the samples. If any samples require further dilution, read the blanks diluted to the same volume as the samples.

NOTE: Contamination can be a problem. Sodium is present everywhere. It is recommended that analysts wear vinyl gloves, not only to avoid contamination but also to protect their hands from HNO_3 . Paper towels, filter papers, detergent, tapwater, water in steam baths, most distilled water, and glass wool all contain significant amounts of Na and must be avoided. The redistilled H_2O used to flush the burner system every 5 to 10 samples (just prior to checking a standard) should be changed frequently. Significant drifting of zero can occur if the burner head gets dirty. Air drifts can cause the signal to be noisy due to temperature changes in the flame. Protect burner from drafts if this becomes a problem.

DETERMINATIVE METHOD

G. CALCULATIONS

1. Procedure

Read results in emission units (X). Subtract blank emission from all samples (K blank should read 0.00 to 0.02; Na blank should read 0.00 to 0.025). Calculate the Na/K concentration (Y) by using the power curve fit formula, $Y = aX^b$, as below. For power curve fits, both X_i and Y_i must be positive. Convert Na/K concentration to desired units, e.g., %, ppm, mg/100, etc., using the appropriate conversion tables.

Power curve fit:

(Concentration) Y

$$Y = aX^b$$

X (Emission)

$$b = \frac{\sum (\ln X_i)(\ln Y_i) - \frac{(\sum \ln X_i)(\sum \ln Y_i)}{n}}{\sum (\ln X_i)^2 - \frac{(\sum \ln X_i)^2}{n}}$$

$$a = \exp[\sum \ln Y_i - (b)(\sum \ln X_i)]$$

where:

X_i = emission of each standard

Y_i = concentration of each standard

$$\Sigma = \sum_{X_i, Y_i}^{X_n, Y_n}$$

n = number of standards used

ln = log normal

Y = sample concentration

X = sample emission

2. Reference

Official Methods of Analysis of the Association of Official Analytical Chemists, 15th Edition.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Determination of Sodium and Potassium in Meat by Atomic Emission Spectroscopy		
2. Protective Equipment	Safety glasses, plastic gloves, and lab coat.		
3. Procedure Steps		<u>Hazards</u>	<u>Recommended Safe Procedures</u>
	B.1 Apparatus		
	I. Glassware: All glassware must be cleaned with 50% HNO ₃ and rinsed with H ₂ O...	Nitric acid is very corrosive and can cause severe eye and respiratory irritation.	This step in the procedure presents the greatest hazard. It should be performed in a fume hood that contains a distilled water tap and a sink. The HNO ₃ bath should be set inside the hood. Then the pieces of glassware may be acid-washed and rinsed, using tongs.
	F.1 Wet Ashing		
	c.1 Add about 10 mL pet ether and warm on steam bath or explosion-proof hot plate...	Petroleum ether is extremely flammable.	This step must be performed in a fume hood. A steam bath is much more desirable for warming, but if an explosion-proof hot plate must be used, avoid heating too rapidly.
4. Disposal Procedure	HNO ₃	See above	May be diluted by slowly adding the acid to the water with stirring and subsequently flushing down an acid-resistant disposal sink with large amounts of water.

SALT

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D
A
-

DETERMINATIVE METHOD

A. INTRODUCTION

Theory

The sodium chloride content is determined by the well-known Volhard method. The sample is treated with AgNO_3 , then wet-ashed, and the excess AgNO_3 is back-titrated with KCNS .

This method embodies many interesting principles of inorganic analytical chemistry. The AgNO_3 solution must be added first, followed by the concentrated HNO_3 . *This order of addition is critical to ensure complete precipitation of the chlorides.* If HNO_3 is added first, loss of chloride by volatilization as HCl could occur because HCl has higher vapor pressure than HCO_3 .

The volume of AgNO_3 solution added must be in excess of that required to react with the chlorides in the sample.

The concentrated solution of KMnO_4 is added to oxidize any organic matter not disposed of by the HNO_3 . Should too much KMnO_4 be accidentally added, the addition of small quantities of sugar will cause color removal.

Following boiling, cooling, and dilution, back-titrate the excess AgNO_3 with KCNS solution, employing ferric ammonium sulfate solution as an indicator.

The $\text{FeNH}_4(\text{SO}_4)_2$ reacts with an excess of thiocyanate, forming the salmon-colored complex, ferric thiocyanate FeCNS^{++} , indicating the end point.

NOTE: After all the silver has been back-titrated, an excess of thiocyanate may react with the precipitated AgCl because the solubility product of AgCNS is 1/100 that of AgCl .

$$^s\text{AgCNS} = 1.0 \times 10^{-12}$$

$$^s\text{AgCl} = 1.1 \times 10^{-10}$$

The addition of nitrobenzene or diethyl ether overcomes this difficulty by coating the precipitated AgCl , thereby withdrawing it from the action of the thiocyanate solution. If results are rounded to 0.1%, precipitate coating is not needed.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

**Reagent and
Solution List**

-
- a. Ferric alum indicator—Saturated aqueous solution of reagent grade $\text{FeNH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$.
 - b. Silver nitrate—0.100N—Dissolve 17.04 g of AgNO_3 , previously dried at 110°C , in distilled water, and dilute to 1 L. Standardize (using excess AgNO_3) against 0.100N NaCl (5.845 g/L) according to Volhard or Mohr method.
 - c. Potassium thiocyanate—0.100N—Dissolve 9.72 g of reagent grade KCNS in distilled water, and dilute to 1 L. Verify the strength of this solution as follows: Pipette 25 mL of standard AgNO_3 solution into a 300 mL Erlenmeyer flask, add 80 mL of distilled H_2O , 15 mL of 1 + 1 HNO_3 , and 2 mL of the ferric alum indicator. Titrate with KCNS solution to a permanent light brown (salmon-colored) end point. The ratio of the volume of KCNS to the volume of AgNO_3 should be 1:1.
 - d. Potassium permanganate—5% aqueous solution. (KMnO_4)
 - e. Diethyl ether—reagent grade.
 - f. Nitric acid—reagent grade. (HNO_3)
-

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE

Determination

-
- a. Weigh 2.5-3.0 g of finely comminuted and thoroughly mixed sample into a 300 mL Erlenmeyer flask.
 - b. Add 25.0 mL of 0.100N AgNO_3 solution, swirl flask until sample and solution are in intimate contact, and then add 15 mL of conc. HNO_3 .
 - c. Add sufficient boiling chips and boil until meat dissolves; add KMnO_4 in small portions; continue boiling until color disappears and solution becomes almost colorless.
 - d. Add 25 mL of distilled H_2O , boil for 5 min, cool to room temperature, and dilute to ca. 150 mL with distilled H_2O .
 - e. Add ca. 5 mL of diethyl ether (optional), 2 mL of the ferric alum indicator, and shake vigorously to coagulate the precipitated AgCl . (If results are rounded to 0.1%, the diethyl ether need not be added.)
 - f. Titrate the excess AgNO_3 with KCNS solution to a permanent, salmon-colored end point.
-

DETERMINATIVE METHOD

G. CALCULATIONS

1. Procedure

$$\text{Percent NaCl} = \frac{[25.0 \text{ mL} - (\text{mL KCNS})(R)](N \text{ AgNO}_3)(5.85)}{\text{Sample Weight}}$$

where R = ratio $\frac{\text{AgNO}_3}{\text{KCNS}}$ as determined in C. Reagents and Solutions.

2. Reference

Official Methods of Analysis of the Association of Official Analytical Chemists,
15th Edition: 935.47, 941.18.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Salt determination.		
2. Required Protective Equipment	Safety glasses, safety shoes, heat-resistant gloves, plastic gloves, and lab coat.		
3. Procedure Steps		<u>Hazards</u>	<u>Recommended Safe Procedures</u>
	F. Determination. b.		
	...add 15 mL of conc. HNO_3 .	Harmful fumes and thermal burns.	This step and each succeeding step in this section should be performed in a fume hood.
4. Disposal Procedures	Acid digestion mixture	Same as above.	Flush with large quantities of water into an acid resistant disposal sink. The area should be well ventilated.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standard

<i>Compound</i>	<i>Analytical Range (%)</i>	<i>Repeatability % CV</i>	<i>Reproducibility % CV</i>
Salt	‡	<0.16*	<0.16*
‡ Limit may vary due to sample and aliquot sizes and sample type. * Standard deviation.			

2. Critical Control Points and Specifications

<i>Record</i>	<i>Acceptable Control</i>
Volhard: Standard silver nitrate	Record all standardization calculations in "Standards Book." 0.1000N \pm 0.0005N.
Standard potassium thiocyanate	Same as above.
Burets	Calibrated at 10 mL intervals. Record in "Standards Book."
Sample size	2.5 g-3.0 g (Smaller sample sizes may be taken for high salt content samples.)
Addition of silver nitrate and nitric acid	Silver nitrate must be added first.
Use of permanganate	Must be used.
Use of precipitate "coater"	Use diethyl ether if salt is to be reported to two or more decimal places—not required if rounding to 0.1% salt. Use safety precautions.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

3. Readiness To Perform

-
- a. Familiarization.
 - i. Phase I: Standards—Not applicable unless performing reagent standardizations.
 - ii. Phase II: Known samples (results from accredited analyst).
 - iii. Phase III: Check samples for analyst accreditation.
 - b. Acceptability criteria.

See section J.1 above.
-

4. Intralaboratory Check Samples

-
- a. System, minimum contents.
 - i. Frequency: 1 per week, not to exceed 20% of samples.
 - ii. Blind samples or random replicates chosen by the supervisor after initial analysis.
 - iii. Records are to be maintained by the analyst and reviewed by supervisor and Laboratory QA Officer.
 - b. Acceptability criteria: refer to J.1 above.

If unacceptable values are obtained, then:

 - i. Stop all official analyses by that analyst.
 - ii. Investigate and identify probable cause.
 - iii. Take corrective action.
 - iv. Repeat Phase III of section J.3 above if cause was analyst-related.
-

5. Interlaboratory Check Sample Program

-
- a. Laboratory responsibility: Analyze using official method and report results promptly.
 - b. Chemistry Division responsibility: Review data and report promptly to participants.
 - c. Acceptability criteria.

See Section J.4 above.
-

6. Sample Acceptability and Stability

-
- a. Matrix: Fresh and processed meat and meat products.
 - b. Sample receipt size, minimum: 500 g.
 - c. Condition upon receipt: Sealed, unspoiled.
 - d. Sample Storage:
 - i. Time: 1 week at 4° C; 3 years at -20° C.
 - ii. Condition: ~ 4° C; -20° C.
-

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

7. Sensitivity

-
- a. Lowest detectable level (LDL): NA.
 - b. Lowest reliable quantitation (LRQ): 0.05%.
 - c. Minimum proficiency level (MPL): 0.05%.
-

SOY FLOUR/CONCENTRATE

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DETERMINATIVE METHOD

A. INTRODUCTION

Theory

Soybean flour (50% protein) and soy protein concentrate (70% protein) are added to meat food products as binders. This method utilizes the fact that dilute acid will dissolve the hemicelluloses of soybean flour and soy protein concentrate but will not affect the cereal flour starch. Soybean flour or soy protein concentrate can be determined, therefore, in the presence of cereal flour.

If a meat food product is heated in an alcoholic solution of caustic potash, the fat is saponified and the protein hydrolyzed. This treatment renders the major constituents of meat (fat, protein, and water) soluble in the medium. Spices, cellulose, and starch (from cereal, if present) remain as sediment.

Dilute acid is then used to dissolve the soybean flour hemicelluloses, which are subsequently reprecipitated with 95% ethanol and quantitated after careful centrifugation. Quantitation is accomplished by means of empirical factors: 6.0 for soybean flour and 2.5 for soy protein concentrate.

It is imperative to adhere closely to the time and speed of centrifugation because of the empirical nature of this determination.

DETERMINATIVE METHOD

B. EQUIPMENT

Apparatus

-
- a. Centrifuge.
 - b. Centrifuge tubes, Goetz, 100 mL, Corning #8220, or equivalent.
 - c. Steam bath.
-

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

**Reagent and
Solution List**

-
- a. Ethanol, 95%.
 - b. Alcoholic caustic potash solution (8%): Dissolve 40 g of KOH in 300 mL of 95% ethanol, and dilute to 500 mL with 95% ethanol.
 - c. Dilute hydrochloric acid—1 + 3: Mix 1 volume of concentrated HCl with 3 volumes of distilled H₂O.
-

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE

Determination

- a. Weigh 10.0 g of sample into a 100 mL Goetz tube.
- b. If corn syrup, corn syrup solids, nonfat dry milk, and/or calcium-reduced dry skim milk are present, extract with two successive 50 mL portions of warm, distilled H₂O. Shake, centrifuge, decant, and discard the supernatant liquid after each extraction. (If CS, CSS, NFDM, or CRDSM are absent, this extraction is to be omitted).
- c. Add 50 mL of 8% alcoholic KOH solution and heat on steam bath with occasional stirring just until meat is digested (15-30 min).
- d. Shake well and centrifuge at 2000 rpm for 4 min. Decant and discard supernatant solution.
- e. Wash residue with 25 mL of 95% ethanol, stirring sediment thoroughly. Centrifuge and decant, discarding alcoholic solution.
- f. Add 50 mL of 1 + 3 HCl, mix thoroughly, stopper, and shake for 1 min. Centrifuge at 2000 rpm for 4 min. (Retain residue for cereal determination).
- g. If supernatant is not clear, filter it through a double thickness #541 Whatman paper or equivalent.
- h. Transfer 25 mL of clear supernatant to a second Goetz tube containing 75 mL of 95% ethanol, shake well, and allow to stand for 1 hour.
- i. Centrifuge for exactly 2 min at speed (rpm) that will deliver a "g force" at the furthest inside tip of the tube equivalent to 480, accelerating to that speed in 1 min.

$$g \text{ force} = (1.118 \times 10^{-5}) (\text{radius of rotation in centimeters}) (\text{speed})^2$$

radius of rotation = distance from center shaft to the furthest inside tip of tube.

Derivation

$$f_{\text{cm/sec}^2} = \text{centrifugal field as cm/sec}^2 = X\omega^2$$

X = radius in cm

ω = speed radians/sec

$$1 \text{ revolution} = 2\pi \text{ radians}$$

$$\text{so, rpm } \frac{2\pi}{60} = \frac{\text{radians}}{\text{sec}} \quad (.10472)(\text{rpm})$$

$$\text{and squaring, } (\text{radians/sec})^2 = (.010966)(\text{rpm})^2$$

$$f_{\text{cm/sec}^2} = (X)(.010966)(\text{rpm})^2$$

$$g = 980.6 \text{ cm/sec}^2$$

$$\text{so, } g = \frac{(X)(.010966)(\text{rpm})^2}{980.6} = (1.118 \times 10^{-5})(X)(\text{rpm})^2$$

DETERMINATIVE METHOD

G. CALCULATIONS

Procedure

Percent soybean flour = volume (mL) of sediment \times 6

Percent soy protein concentrate = volume (mL) of sediment \times 2.5

NOTE: The sediment remaining from the dilute HCl leaching may be used to determine cereal content, if present. Decant the HCl, mix residue with 50 mL of 1 + 1 HCl, and proceed as under gravimetric section of CRL method.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Soybean Flour and Soybean Protein Concentrate Determination.		
2. Required Protective Equipment	Safety glasses, plastic gloves, and lab coat.		
3. Procedure Steps		<u>Hazards</u>	<u>Recommended Safe Procedures</u>
	C. Reagents		
	Determination— (c) Add 50 mL of 8% alcoholic KOH solution—etc.	Skin and eye irritation	Procedure should be performed in an efficient fume hood.
	Determination— (e) Wash the residue with 95% ethanol,—etc.	Dermal and respiratory irritation	In addition to using an efficient fume hood, it would be desirable to locate the centrifuge within arms reach of the hood.
4. Disposal Procedures	Alcoholic KOH and sample digest solution	Skin and eye irritation.	Flush into disposal sink with large quantities of water.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN

1. Performance Standard

<i>Compound</i>	<i>Analytical Range (%)</i>	<i>Repeatability % CV</i>	<i>Reproducibility % CV</i>
Soya flour and concentrate	‡	<15.0	<20

‡ Limit may vary due to sample and aliquot sizes and sample type.

2. Critical Control Points and Specifications

<i>Record</i>	<i>Acceptable Control</i>
Dilute hydrochloric acid	Must be 1 + 3 only.
Sample size	10 g \pm 0.1 g.
Removal of CSS and NFDM	Check for presence via label or screening test and remove if present.
Standing time for final precipitate	1 hr \pm 5 min.
Final centrifugation	2 min \pm 5 sec at "g force" 480; accelerate to that speed in 1 min \pm 5 sec (requires accurate tachometer and timer).
Calculations	Recheck; use proper factor for type of soya present.

3. Readiness To Perform

- a. Familiarization.
 - i. Phase I: Standards—NA.
 - ii. Phase II: Fortified samples. Replicates of fortified fresh product and cooked sausage—2 each on 3 days.
 - iii. Phase III: Check samples for analyst accreditation. Random replicates selected by supervisor.
- b. Acceptability criteria.

See section J.1 above.

DETERMINATIVE METHOD

J. QUALITY ASSURANCE PLAN (Continued)

4. Intralaboratory Check Samples

-
- a. System, minimum contents.
 - i. Frequency: Not to exceed 20% of samples.
 - ii. Blind samples or random replicates chosen by supervisor after initial analysis.
 - iii. Records are to be maintained by analyst and reviewed by the supervisor and Laboratory QA Officer.
 - b. Acceptability criteria.

If unacceptable values are obtained, then:

 - i. Stop all official analyses for that analyst.
 - ii. Investigate and identify probable cause.
 - iii. Take corrective action.
 - iv. Repeat Phase III of section J.3 if cause was analyst-related.
-

5. Sample Acceptability and Stability

-
- a. Matrix: Meat, poultry, and meat and poultry products.
 - b. Sample receipt size, minimum: 1 lb.
 - c. Condition upon receipt: Cold (4° C), sealed against evaporation.
 - d. Sample storage:
 - i. Time: Up to 3 years.
 - ii. Condition: - 20° C.
-

6. Sensitivity

-
- a. Lowest detectable level (LDL): NA.
 - b. Lowest reliable quantitation (LRQ): 0.50 SPC/1.20 SF
 - c. Minimum proficiency level (MPL): 0.25 SPC/0.60 SF.
-

SOY FLOUR/CONCENTRATE (MICRO ID)

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J. [Reserved]	

DETERMINATIVE METHOD

A. INTRODUCTION

Theory

The presence of characteristic "hour glass" or I-shaped cells (sometimes called "bearer cells") in meat or meat food products is an indication of the presence of soya flour or concentrate in those products. Because of modern refinement techniques in the processing of soya beans for flour and soya protein concentrate, these "hour glass" cells are often broken into small fragments that are not easily identified upon microscopic examination. This procedure, therefore, is limited in its usefulness.

SOY2
May, 1993

PS12DETERMINATIVE METHOD

B. EQUIPMENT

Apparatus

-
- a. 250 mL beaker.
 - b. Glass stirring rod.
 - c. Steam bath.
 - d. Centrifuge tubes, Goetz, 100 mL, Corning #8220, or equivalent.
 - e. Centrifuge.
 - f. Microscope (100x), preferably equipped with polarized light accessories.
-

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and
Solution List

-
- a. 8% alcoholic KOH solution; w/v: Dissolve 40 g of KOH in 300 mL 95% ethanol and dilute to 500 mL with 95% ethanol.
 - b. 95% ethanol.
 - c. Concentrated HCl.
 - d. 25% ethanol in H₂O, v/v; prepared with reagent b.
-

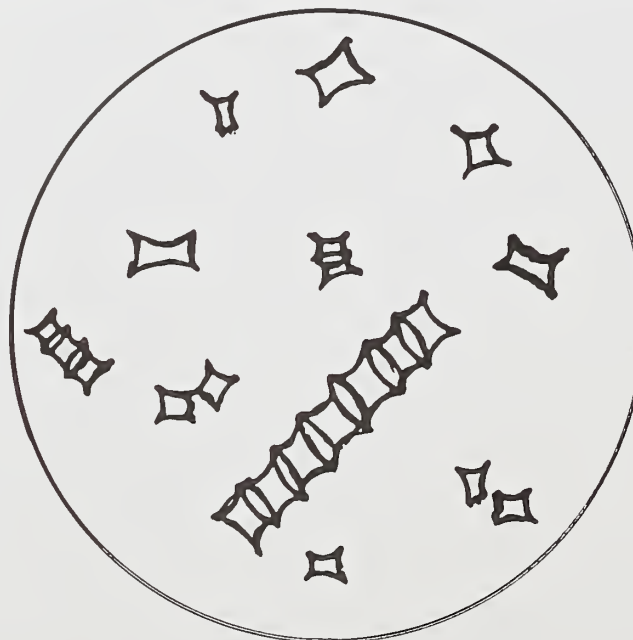
DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE

1. Determination

- a. Mix 10 g finely divided samples in 250 mL beaker with 75 mL 8% alcoholic KOH solution, and heat on steam bath until all meat is digested (15-30 min).
- b. Transfer liquid and residue to 100 mL Goetz tube with 95% ethanol and let settle.
- c. Decant as completely as possible, and cover residue with ca. 50 mL warm H₂O.
- d. Stopper tube and shake vigorously; let stand a few minutes until foam subsides; then transfer to a second tube and centrifuge.
- e. Pour off and discard supernatant, and add 10 mL concentrated HCl.
- f. Stopper and shake, or mix contents thoroughly with glass rod.
- g. Add ca. 15 mL 25% ethanol, mix, and centrifuge.
- h. Decant supernatant and examine residue under microscope at 100x (or other appropriate magnification) for characteristic "hour glass" cells. Examination under polarized light is preferred because it causes the cells to be more visually perceptible.

2. Example



NOTE: Dry powder mixtures may be examined in the same manner by wetting a small portion on a microscope slide and microscopically examining it as in step h above.

3. Reference

AOAC Official Methods of Analysis, 15th Edition, 1990, 913.01.

DETERMINATIVE METHOD

H. HAZARD ANALYSIS

1. Method Title	Microscopic Identification of Soya Flour and Concentrate.		
2. Required Protective Equipment	Safety glasses, plastic gloves, and lab coat.		
3. Procedure Steps		<u>Hazards</u>	<u>Recommended Safe Procedures</u>
	C. Reagents		
	Determination— (a) Add 75 mL of 8% alcoholic KOH solution—etc.	Skin and eye irritation	Procedure should be performed in an efficient fume hood.
	Determination— (b) Transfer liquid and residue with 95% ethanol,—etc.	Dermal and respiratory irritation	In addition to using an efficient fume hood, it would be desirable to locate the centrifuge within arms reach of the hood.
4. Disposal Procedures	Alcoholic KOH and sample digest solution	Skin and eye irritation.	Flush into disposal sink with large quantities of water.

VITAMIN A

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DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory

Antimony trichloride forms a color complex with both vitamin A and carotene. Column chromatography is used to separate the vitamin A fraction from that of carotene. The Carr-Price reaction product is used to measure vitamin A at 620 nm. Carotene is estimated by its absorption at 436 nm.

2. Applicability

The procedure is applicable for the analysis of total vitamin A (vitamin A plus carotene) in meat and poultry products.

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

NOTE: Vitamin A is light sensitive; to prevent its destruction amber or low-actinic glassware or subdued lighting must be used.

- a. Boiling flask: 250 mL.
- b. Condensers: Air- or water-cooled.
- c. Erlenmeyer flasks: 500 mL; 250 mL.
- d. Separatory funnels: 500 mL.
- e. Volumetric pipettes.
- f. Mohr pipette: 10 mL.
- g. Alundum boiling chips.
- h. Chromatographic tubes: 2.5 cm od × 25.0 cm with sealed-in disc of medium porosity and with funnel on upper end and stem on lower end, 8 mm od × 40 mm (available from SGA, Scientific Inc.)
- i. Ultraviolet lamp: Longwave, 360 nm is recommended. Caution! Vitamin A is destroyed by too intense UV light; use low-intensity lamp.

2. Instrumentation

Spectrophotometer: Suitable for reading at 620 nm and 426 nm.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagents and Solution List

-
- a. Potassium hydroxide solution: Dissolve 90 g KOH in 100 mL.
 - b. Diethyl ether (peroxide free).
 - c. Chloroform (H₂O and phosgene free).
 - d. Hexane.
 - e. Acetone.
 - f. Nitrogen (oxygen free).
 - g. Hyflo Super-Cel.
 - h. Sea Sorb 43.
 - i. Isopropanol.
 - j. Ethanol.
 - k. Antimony trichloride reagent: *Warning! Corrosive Reagent!* Dissolve 114 g SbCl₃ in 500 mL of chloroform. It may be necessary to warm the mixture. Filter and store in dry, glass-stoppered standard taper bottle.
 - l. Acetone-hexane mixture: 50 mL acetone and 450 mL hexane.
 - m. Hyflo Super-Cel:Sea Sorb 43(1:1). Mix 1 lb of each and store.
 - n. B-carotene: Prepare solutions of pure B-carotene in acetone-hexane (1 + 9) just before use for preparation of standard curve (0.6 mg B-carotene is equivalent to 1 International Unit vitamin A).
-

DETERMINATIVE METHOD

D. STANDARDS

1. Preparation

-
- a. Stock solution: Weigh approximately 0.5 g of vitamin A reference standard into a 50 mL volumetric flask. This is the contents of 3 capsules. Make up to volume with isopropanol. One gram of vitamin A reference standard contains 100,000 International Units.
 - b. Working solution: Transfer a 2 mL aliquot of the stock solution to a 200 mL volumetric flask and make up to volume with chloroform. (1 mL = approximately 10 IU.)
-

2. Storage Conditions

Stock and working solutions should be refrigerated.

3. Shelf Life Stability

Stock solution: 1 week.

Working solution should be prepared fresh on the day of use.

DETERMINATIVE METHOD

E. SAMPLE HANDLING

Sample Preparation

NOTE: Protect vitamin A from strong illumination by working in subdued light or by using low-actinic glassware.

From stated label claim, process sufficient sample to contain about 200 IU vitamin A. Frozen products should be first ground by two passes through meat grinder. Grind 200-300 g for sampling. The sample is comminuted in a blender as quickly as possible to prevent oxidation.

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE

Determination

- a. Transfer 20 g of sample to a 250 mL boiling flask with a ground glass neck. Use a powder funnel to keep sample from touching neck and wash in with 50 mL of water.
- b. Add 50 mL of ethanol, 10 mL of the KOH solution, and a few grains of alundum for boiling. Mix sample and reflux for 30 min with occasional shaking. If unsaponified fat is visible at end of digestion period, add another 10 mL aliquot of KOH solution to sample and repeat saponification. Higher KOH:ethanol ratio should be used for all known high fat content samples.
- c. Cool sample, shake with stopper on top to get most of the sample off the sides, and then transfer to a 500 mL separatory funnel. Police flask and transfer all washings to separatory funnel.

NOTE: Use a rubber-tipped glass rod and ethanol to aid in the transfer.

- d. Add 50 mL of ether and 50 mL of hexane to the separatory funnel. Gently invert once and release the pressure from the top. Gently invert once more and release pressure. Sample may now be shaken normally for approximately 1 min.
- e. Transfer the ether-hexane layer into a 500 mL Erlenmeyer flask (first extraction).
- f. Extract the water layer with 50 mL of ether and 50 mL of hexane.
- g. Drain the water and discard.
- h. Combine the extracts in the separatory funnel, washing the 500 mL Erlenmeyer flask with ether. Add wash to separatory funnel.
- i. Add 300 mL of water and gently invert once.

Caution: Do not shake; Vitamin A will be lost.

- j. Drain water layer into 500 mL Erlenmeyer flask and wash the ether layer again with 300 mL of water by inverting one time.
- k. Draw off small portion of water layer into test tube and see if it is neutral to phenolphthalein. If not, wash until neutral, then draw off water layer into 500 mL Erlenmeyer flask. Discard water washings.
- l. Draw off the ether layer into a 250 mL Erlenmeyer flask and place in a fume hood on a hot water bath to drive off the ether.
- m. Blow a stream of oxygen-free nitrogen onto the sample while boiling. This speeds volatilization and keeps Vitamin A from oxidizing. When dry, cover residue with a few mL of hexane.

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE (Continued)

2. Column Preparation

Column should be approximately 25.0 cm long and 2.5 cm in diameter. Place a piece of glass wool over the tip of the column, put a one-hole rubber stopper on the column tip, and place in a 125 mL suction flask.

- a. Pour a 1:1 mixture of the absorbents through a powder funnel into the column. Add enough absorbent to give a height of 12.5 cm and tap lightly.
- b. Pull a vacuum and wet column with hexane. Just before the column goes dry, pour the sample into the column and wash lip and flask with hexane and add to column.
- c. Just before column goes dry, add the eluant (10% acetone-hexane mixture) and observe elution of orange band of carotene. (Carotenes pass through column rapidly.)
- d. Collect entire carotene band. When all the carotene is in the 125 mL suction flask, turn off the vacuum and remove the flask.
- e. Transfer eluate, which has been reduced in volume by loss of vapor through H₂O pump to 100 mL volumetric flask, dilute to volume with acetone-hexane (1 + 9), stopper, and set aside in dark for later analysis.
- f. Mark this flask "carotene fraction."
- g. Place second flask under column and continue eluting until all vitamin A comes off column. Observe elution of vitamin A by brief inspections with UV light (Vitamin A fluoresces under UV light). Mark this flask "vitamin A fraction."

3. Photometer Measurement

- a. Vitamin A fraction.
 - i. The wavelength is set at 620 nm. Instrument should have at least 15-min warmup before use. The instrument is first set to zero on the absorbance scale using a reagent blank. The reagent blank consists of 1 mL chloroform and 3 mL of the SbCl₃ solution.
 - ii. Pipet 1 mL of the standard solution (1 mL = approximately 10 IU) into the cuvette; place cuvette in the photometer; and quickly add 3 mL of the SbCl₃ solution by means of a dispensing pipet. Each standard and each sample must be in duplicate and the average value used for the reading.

NOTE: Vitamin A measurement must be done immediately. Vitamin A starts to deteriorate rapidly.
 - iii. One mL of sample is added to the cuvette and 3 mL of SbCl₃ solution added quickly. The reading is taken and repeated again using the average value. If reading of samples is too low, take more sample; if too high, make a further dilution.

Sample should read within 5-15 International Units.

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE (Continued)

b. Carotene fraction.

Calibrate suitable spectrophotometer with acetone-hexane (1 + 9) solutions of pure B-carotene as shown by characteristic absorption curve (J. Bio Chem 144, 21. 1942). Read absorbance at 436 nm. Prepare calibration chart and convert absorption A of solution to be determined to carotene concentration from chart as mg carotene/mL.

DETERMINATIVE METHOD

G. CALCULATIONS

1. Procedure

US Recommended Daily Allowance (USRDA) = 5000 International Units (IU)

a.
$$\frac{\text{IU vitamin A}}{\text{serving}} = C (\text{std}) \times \frac{A (\text{sample})}{A (\text{std})} \times \frac{V (\text{sample})}{W (\text{sample})} \times F$$

C (std) = Concentration of standard (IU/mL)

A (sample) = Absorbance of sample

A (std) = Absorbance of standard

V = Volume of sample (mL) aliquot

W (sample) = Weight (g) of sample in the aliquot

F = Serving size converted to the appropriate dimensions
(i.e., ounces to grams, etc.)

b. Calculate carotene as units vitamin A per serving.

$$\text{IU Vitamin A (as B-carotene) per serving} = C \times \frac{V}{W} \times F \times 1.667$$

C = mg carotene/mL in 100 mL flask

V = Volume of sample (100 mL)

W = Weight of sample in aliquot (20 g)

F = Serving size converted to appropriate dimensions
(ounces to grams, etc.)

1.667 = Equivalent of carotene to vitamin A

c. Total IU vitamin in sample = 1.a + 1.b

$$\text{USRDA} = \frac{\text{mg vitamin A}^*}{5000} \times 100$$

*IU vitamin A found per serving

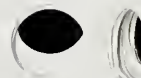
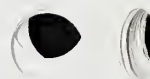
2. Reference

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- Methods of Vitamin Assay, The Association of Vitamin Chemists (1966).
 - Methods of Analysis of the Association of Official Analytical Chemists, 15th Edition.
-

THIAMINE [B₁]

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DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory

Thiamine occurs naturally in foods either in a free or combined form as a protein complex, or as a pyrophosphoric acid ester, cocarboxylase. A deficiency of thiamine results in beriberi, polyneuritis, neuritis of several peripheral nerves, loss of weight and appetite, etc.

Thiamine or thiamine hydrochloride is rapidly destroyed in alkaline or neutral solutions; however, in acid solutions (pH 3.5), the vitamin can be autoclaved at 120° C for 30 min without loss of activity.

The determination of thiamine is based on the oxidation of thiamine to thiochrome, which fluoresces in ultraviolet light. In the absence of other fluorescing compounds, the fluorescence is proportional to the amount of thiochrome present, and therefore the amount of thiamine in a sample.

Thiamine must be freed from interfering substances by elution from an appropriate absorption column.

2. Applicability

This procedure is applicable to meat or poultry products.

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

-
- a. Volumetric flasks: 100 mL.
 - b. Powder funnels.
 - c. Volumetric pipets.
 - d. Erlenmeyer flasks: 125 mL.
 - e. Standard taper glass-stoppered graduated cylinders.
 - f. Standard taper glass-stoppered 50 mL centrifuge tubes.
-

2. Instrumentation

-
- a. Photofluorometer: Fitted with input filter of narrow transmittance range with maximum ca. 365 nm and an output filter of narrow transmittance range with maximum at ca. 435 nm.
 - b. Chromatographic columns: Base exchange tubes with reservoir at top approximately 35 mm od × 95 mm, adsorption tube mid-section 8 mm od × 145 mm, drawn into a capillary at the bottom 35 mm long which is restricted to permit a flow of 1 mL/min. (Tubes are commercially available as base exchange tubes.)
-

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and
Solution List

-
- a. Anhydrous sodium sulfate, granular.
 - b. 15% sodium hydroxide solution: Dissolve 150 g NaOH in water and dilute to 1 L.
 - c. 1% potassium ferricyanide solution: Dissolve 5 g of $K_3Fe(CN)_6$ in water and dilute to 500 mL. Stable indefinitely if kept cool and dark in brown bottle.
 - d. Alkaline potassium ferricyanide solution—Dilute 3 mL of 1% $K_3Fe(CN)_6$ to 100 mL with 15% NaOH solution. Prepare fresh daily and keep out of sunlight.
 - e. 0.1N H_2SO_4 solution: Dilute 2.8 mL of conc. H_2SO_4 to 1 L with water.
 - f. 2.5M sodium acetate solution: Dissolve 345 g of $NaC_2H_3O_2 \cdot 3H_2O$ in water and dilute to 1 L.
 - g. Isobutyl alcohol: Check fluorescence; should be $\leq 10\%$ of quinine standard.
 - h. Enzyme solution: Prepare fresh daily. Dissolve one of the following enzymes in 50 mL of 2.5M sodium acetate: (1) 2 g Takadiastase, (2) 6 g Prolidase S, or (3) 2 g Mylase P.
 - i. Acid 25% potassium chloride solution: Dissolve 250 g of KCl in water, add 8.5 mL conc. HCl, and dilute to 1 L with water.
 - j. 3% KCl solution: Dilute 25 mL of 25% KCl solution to 200 mL with 0.1N HCl.
 - k. Activated Decalso (Thiochrome Decalso): Add 4 g per column of Decalso to a 400 mL beaker. Wash twice with hot 3% KCl (Keep KCl in contact with Decalso for 10 min), then with hot distilled water until free of chlorides. Check with 1% $AgNO_3$.
 - l. Stock quinine sulfate solution: Dissolve 100 mg in 0.1N H_2SO_4 and dilute to 1 L with the same solvent. Stable indefinitely in dark brown bottle.
 - m. Working quinine sulfate solution: Dilute 3 mL of stock solution to 1 L with 0.1N H_2SO_4 .
-

DETERMINATIVE METHOD

D. STANDARDS

1. Preparation

-
- a. Stock thiamine solution: Dry thiamine hydrochloride over P_2O_5 for 24 hours. Dissolve 100 mg in 0.01N HCl and make to 1 L volume with same.
 - b. Intermediate thiamine solution: Dilute 5 mL of stock solution to 100 mL with water.
 - c. Working thiamine solution 0.2 μ g/mL: Transfer 4 mL of intermediate thiamine solution to a flask containing 75 mL of 0.1N H_2SO_4 and 5 mL of sodium acetate solution and adjust to 100 mL with water.
-

2. Storage Conditions

Stock thiamine solution should be stored at 5° C in dark bottle.

3. Shelf Life Stability

Stock thiamine solution is stable up to 6 months if stored as described above. Working thiamine solution should be prepared fresh on the day of use.

DETERMINATIVE METHOD

E. SAMPLE HANDLING

Sample Preparation

The sample must be comminuted in a blender before it can be used. To do this, weigh 300 g of sample and 300 g of water into a blender and comminute.

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE

Determination

- a. Weigh 20 g of the slurry (10 g sample) into a 125 mL low-actinic Erlenmeyer flask.
 - b. Add 7.5 mL of 1N HCl and 40 mL of distilled water.
 - c. Add 2 drops of caprylic alcohol to prevent foaming and place in autoclave for 15 min at 15 lb pressure.
 - d. Cool to room temperature and add 5 mL of the enzyme solution to each sample.
 - e. Incubate for 2 hr at 45° C. At this point, the sample has a pH of 4.5 and is stable for 2 weeks in a refrigerator.
 - f. Bring sample to 100 mL with water and filter through a Whatman #4 paper.
 - g. Prepare columns using the activated Decalso. Wash twice with hot 3% acidified KCl and then with hot distilled water until clear of chlorides. Wash once with distilled water to cool columns. Always keep water above the Decalso in the column.
 - h. Pass 50 mL of the filtered sample through the column. This may have to be done with two 25 mL aliquots. After sample has passed through the column, wash twice with distilled water to remove any extraneous matter in column.
 - i. Elute column with 25 mL (10, 10, 5 mL aliquots) of 25% acidified KCl solution into a ground glass graduated cylinder. *You can stop at this point and refrigerate.*
 - j. A standard of 0.2 µg/mL should be prepared and 25 mL passed through the column the same as the sample. If solution in graduate is not 25 mL, make the 25 mL with water.
 - k. Only two reaction vessels can be used at a time. Into one 50 mL centrifuge tube, pipette 5 mL of standard and into the other, pipette 5 mL of sample. To each tube add 3 mL of alkaline ferricyanide solution and mix gently; then add 20 mL of isobutyl alcohol. Place on the shaker and shake for exactly 90 sec. Centrifuge at 2500 rpm for 60 sec. Remove and discard lower aqueous phase using syringe (approximatley 9 mL). Add a heaping spatula (ca. 2 g) of sodium sulfate to the isobutyl layer and centrifuge for 60 sec.
 - l. Adjust meter reading on photoflurometer to 60, using quinine sulfate as the set point solution.
 - m. Pour standard (0.2 µ/mL) into cuvette and read. Add 3 drops of (1:1) HCl, mix, and read standard blank. Do the same with the samples.
-

DETERMINATIVE METHOD

G. CALCULATIONS

1. Procedure

United States Recommended Daily Allowance (USRDA) = 1.5 mg

$$\frac{A_{sample} - A_{blank}}{A_{std} - A'_{blank}} \times C_{std} \times \frac{Df}{1000} \times F = \text{mg/serving}$$

$$\% \text{ USRDA} = \frac{\text{mg/serving}}{1.5} \times 100$$

A_{sample} = Fluorescence of sample

A_{blank} = Fluorescence of sample blank

A'_{sample} = Fluorescence of standard blank

A_{std} = Fluorescence of standard

C_{std} = Concentration of standard (0.2 $\mu\text{g/mL}$)

Df = Dilution factor; if above dilutions are used, the factor

$$\frac{25 \text{ mL}}{50 \text{ mL}} \times \frac{100 \text{ mL}}{10 \text{ g}} = 5 \text{ mL/g}$$

F = Serving size converted to the appropriate dimensions
(i.e., ounces to grams, etc.)

1000 = micrograms per milligram

2. Reference

Methods of Vitamin Assay, The Association of Vitamin Chemists (1966).

RIBOFLAVIN [B₂]

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DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory

Riboflavin is a naturally occurring pigment that has wide distribution in plant and animal cells. In living cells riboflavin is usually combined with phosphoric acid or with phosphoric acid and adenylic acid (adenosine monophosphate), both of which may be combined with specific proteins.

Because it may be combined with proteins, it is necessary to treat natural products with acid or enzymes to free the riboflavin from its protein combination and make it available for extraction.

Riboflavin fluoresces in light of wave length 440 to 300 nm. The intensity of fluorescence is proportional to the concentration of riboflavin. The riboflavin is determined by the difference between its fluorescence before and after chemical destruction. All readings are taken on one aliquot to compensate for the variations due to the presence of interfering substances. Interfering substances that fluoresce may be removed by adsorption chromatography or oxidation or both.

2. Applicability

This procedure is applicable to meat or poultry products. The sample preparation is similar to that for vitamin B₁ and a suitable aliquot from the B₁ sample preparation may be used in this determination.

DETERMINATIVE METHOD

B. EQUIPMENT

1. Apparatus

All glassware should be low-actinic.

- a. Test tubes: 18 mm od × 150 mm.
- b. Funnels: 2-3 inches diameter.
- c. Erlenmeyer flasks, low-actinic: 125 mL.
- d. Filter paper: Whatman No. 541.
- e. Volumetric flasks: 25 mL, 100 mL.
- f. Volumetric pipettes: Assorted sizes.

2. Instrumentation

Photofluorometer: Use fluorometer suitable for accurately measuring fluorescence of solutions containing riboflavin in concentrations of 0.05-0.2 $\mu\text{g/mL}$. Instrument should be fitted with input filter of narrow transmittance range with maximum at ca. 440 nm and an output filter of narrow transmittance range with maximum at ca. 565 nm.

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

**Reagent and
Solution List**

- a. Sodium acetate 2.5M: Dissolve 340 g of sodium acetate in water and make up to 1 L.
 - b. Enzyme solution: Prepare fresh daily. Dissolve one of the following enzymes in 50 mL of 2.5M sodium acetate: (1) 2 g Takadiastase, (2) 6 g Prolidase S, or (3) 2 g Mylase P.
 - c. Glacial acetic acid.
 - d. 1N hydrochloric acid: Add 83 mL of concentrated HCl to water and make up to 1 L.
 - e. 4% KMnO_4 solution: Dissolve 4 g of KMnO_4 in water and make up to 100 mL. Solution is stable for one week.
 - f. Caprylic alcohol.
 - g. 3% hydrogen peroxide.
 - h. Sodium fluorescein solution (0.05 $\mu\text{g/mL}$): Weigh 0.05 g of sodium fluorescein into a 1 L volumetric flask and make to volume with water. Take a 1 mL aliquot and make to 1 L with water (0.05 $\mu\text{g/mL}$).
 - i. Sodium hydrosulfite (Dithionite): High purity and stored to avoid undue exposure to light and air.
-

DETERMINATIVE METHOD

D. STANDARDS

1. Preparation

-
- a. Stock solution (25 $\mu\text{g/mL}$): Weigh accurately 25 mg U.S.P. Reference standard riboflavin dried over P_2O_5 into a 1 L volumetric flask. Add 700 mL of water, 1.2 mL of glacial acetic acid, and warm to aid solution. Cool and make up to volume with water.
 - b. Riboflavin standard A (5 $\mu\text{g/mL}$): Dilute 20 mL of stock solution to 100 mL with water.
 - c. Riboflavin working standard B (0.5 $\mu\text{g/mL}$): Dilute 10 mL of stock solution A to 100 mL with water using 7.5 mL of 1N HCl, 5 mL of 2.5M NaOAC, and make to volume with water.
-

2. Storage Conditions

Preserve under toluene, protect from light in a refrigerator.

3. Shelf Life Stability

Stored under the above conditions, stock solution is good for six months; riboflavin standard A solution is good for two weeks; and riboflavin working standard B is stable for one week.

DETERMINATIVE METHOD

E. SAMPLE HANDLING

Sample Preparation

The sample must be comminuted in a blender before it can be used. To do this, weigh 300 g of sample and 300 g of H₂O into a blender and comminute.

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE

Determination

- a. Weigh 20 g of the slurry into a 125 mL low-actinic Erlenmeyer flask.
 - b. Add 7.5 mL of 1N HCl and 40 mL of distilled water.
 - c. Add 2 drops of caprylic alcohol to prevent foaming and place in autoclave for 15 min at 15 lb pressure. The 20 g sample weighed consists of 10 g sample and 10 g water. Keep this in mind for calculations.
 - d. Cool to room temperature and add 5 mL of the enzyme solution to each sample.
 - e. Incubate for 2 hr at 45° C. At this point, the sample has a pH of 4.5 and is stable for 2 weeks in a refrigerator.
 - f. Transfer sample to a 100 mL volumetric flask and bring to volume with water. From this sample preparation aliquots for both riboflavin and thiamine determinations may be taken.
 - g. Filter sample through Whatman #4 or #541 paper. Transfer a 15 mL aliquot to a 25 mL low-actinic volumetric flask.
 - h. Add 1 mL of glacial acetic acid, 1 mL of a 4% KMnO₄ solution, and allow to stand exactly 2 min.
 - i. Add 5 mL of 3% H₂O₂ and mix thoroughly. Add two drops of caprylic alcohol and bring to volume.
 - j. Using the 0.05 µg/mL sodium fluorescein, adjust the instrument to give a deflection of 50 on the meter. Check adjustment before reading each series.
 - k. Darken lights in the room. Transfer a 10 mL aliquot to a test tube.
 - l. Pour sufficient sample from the test tube to the cuvette and read fluorescence.
 - m. Pour sample back into the test tube. Add a 1 mL aliquot of the riboflavin working standard, mix, and read in fluorometer.
 - n. Pour back in test tube, add ca. 10 mg of sodium hydrosulfite, mix, and read fluorescence.
-

DETERMINATIVE METHOD

G. CALCULATIONS

1. Procedure

United States Recommended Daily Allowance (USRDA) = 1.7 mg

$$\frac{A - 1.10C}{1.10B - A} \times \frac{\text{Riboflavin increment}}{10} \times \frac{Df}{1000} \times F = \text{mg/serving}$$

$$\% \text{ USRDA} = \frac{\text{mg/serving}}{1.7} \times 100$$

A = sample absorbance

B = sample + standard absorbance

C = absorbance after addition of sodium hydrosulfite

10 = sample aliquot (mL)

1000 = micrograms/milligram

Df = Dilution factor; if above dilutions are used, factor 16.7 mL/g

1.10 = Decimal equivalent of 11/10 (10 mL sample aliquot plus 1 mL riboflavin working standard)

F = Serving size converted to the appropriate dimensions
(i.e., ounces to grams, etc.)

Riboflavin increment = 0.5 $\mu\text{g/mL}$ —if method is followed

2. Reference

Methods of Vitamin Assay, The Association of Vitamin Chemists (1966).

VITAMIN C/TITRATION

Contents

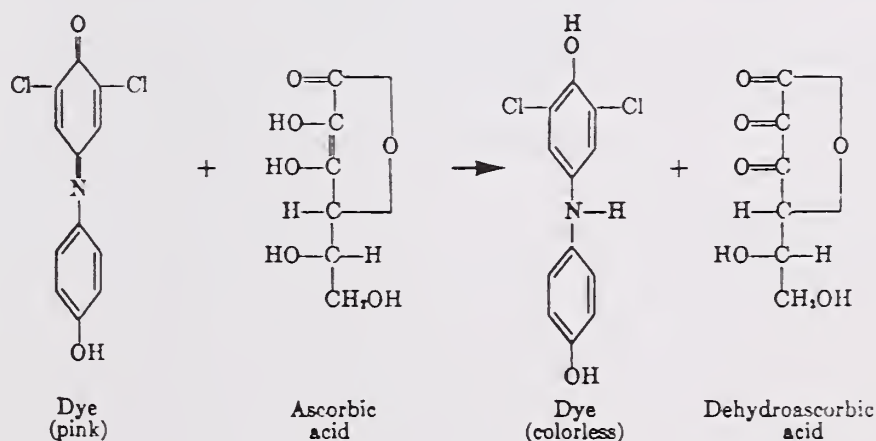
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DETERMINATIVE METHOD

A. INTRODUCTION

1. Theory

The oxidation-reduction properties of ascorbic acid are widely used as the primary reaction for the determination of vitamin C. The reducing property of vitamin C is measured by using a suitable oxidizing agent such as 2,6-dichlorophenol indophenol. The following equation illustrates this reaction:



In this procedure, the dye is made up in a dilute sodium bicarbonate solution and is blue, but in acid medium the dye is pink.

The following compounds may be analyzed by this procedure: ascorbic acid, also known as "vitamin C"; sodium ascorbate; d-isoascorbic acid, also known as erythorbic acid; and sodium d-iso ascorbate, also known as sodium erythorbate.

2. Applicability

This method is applicable to the determination of reduced ascorbic acid and its sodium salt, and d-isoascorbic acid (erythorbic acid) and its sodium salt in meat and poultry products.

DETERMINATIVE METHOD

B. EQUIPMENT

Apparatus

-
- a. Blender.
 - b. Funnel, 3" diameter.
 - c. Filter paper.
 - d. Volumetric pipette, 25 mL.
 - e. Burette, 50 mL capacity.
 - f. Stop watch.
 - g. Magnetic stirrer and stirring bars.
-

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

Reagent and
Solution List

-
- a. Indophenol dye: Add 105 mg NaHCO_3 and 125 mg 2,6-dichlorophenol indophenol sodium salt to 125 mL distilled water. Shake until dissolved and dilute to 500 mL. Store in amber bottle in refrigerator. Prepare weekly.

NOTE: The 2,6-dichlorophenol indophenol dye is reduced equally well by the enediol groups of ascorbic acid, sodium ascorbate, isoascorbic acid, and sodium isoascorbate, so that this method is applicable to all four compounds and cannot distinguish among them.

- b. Metaphosphoric acid: Dilute 15 g metaphosphoric acid in 40 mL glacial acetic acid to 500 mL with distilled water.
-

DETERMINATIVE METHOD

D. STANDARDS

Preparation of Standards

-
- a. 1 mg/mL ascorbic acid stock solution: Add 100 mg ascorbic acid to 1 mL glacial acetic acid. Dilute to 100 mL with distilled water. Make fresh daily.
 - b. 40 μ g/mL ascorbic acid working solution: Add 4 mL of stock solution to 96 mL distilled water. Make just before use.
-

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE

Determination

-
- a. Blend 10 g of product with 95 mL metaphosphoric acid (refer to section C, Reagent and Solution List, item b) and filter.
 - b. Titrate 25 mL aliquot of filtrate with the indophenol dye solution until pink color remains at least 30 sec.
 - c. Titrate 25 mL aliquot ascorbic acid working solution.
 - d. Titrate 25 mL metaphosphoric acid reagent as a reagent blank.

NOTE: Ascorbic acid solutions are unstable and the presence of oxidizing agents in the meat (e.g., nitrites) cause the solution to decompose rapidly. Therefore, conduct the analysis for ascorbic acid and its related compounds as rapidly as possible.

NOTE: A blank value can be obtained by analyzing untreated meat in the same way to determine the ascorbic acid equivalent of the natural reducing substances present in meat. (For ground beef, this value is 25 ppm calculated as ascorbic acid.)

DETERMINATIVE METHOD

G. CALCULATIONS

1. Procedure

$$\text{ppm ascorbic acid} = \frac{(A - B)C}{D} \times 1000$$

United States Recommended Daily Allowance (USRDA) = 60 mg

$$\text{mg vitamin C per serving} = \frac{(A - B)C}{D} \times F$$

$$\% \text{ USRDA} = \frac{\text{mg vitamin C/serving}}{60} \times 100$$

A = mL sample aliquot titration

B = mL reagent blank titration

$$C = \text{mg ascorbic acid per mL dye} = \frac{1 \text{ mg}}{\text{mL standard aliquot titration}}$$

D = 2.5 g sample set.

F = Serving size converted to the appropriate dimensions (i.e., ounces to grams, etc.)

NOTE: If possible, standardize with the ascorbate compound to be analyzed in the meat sample. The following are equivalents between the various compounds:

1 part ascorbic acid = 1.000 parts erythorbic acid

1 part ascorbic acid = 1.124 parts sodium ascorbate

1 part ascorbic acid = 1.124 parts sodium erythorbate

1 part sodium ascorbate = 1.000 parts sodium erythorbate

1 part erythorbic acid = 1.124 parts sodium erythorbate

2. Reference

Methods of Vitamin Assay, The Association of Vitamin Chemists (1966).

VITAMIN C/SPECTROPHOTOMETRIC

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DETERMINATIVE METHOD

B. EQUIPMENT

Apparatus

-
- a. Blender.
 - b. Volumetric flasks: 25 mL, 50 mL, 100 mL.
 - c. Volumetric pipettes: 3 mL, 5 mL, 10 mL.
 - d. Spectrophotometer: Suitable for reading at 515 nm.
 - e. Erlenmeyer flasks: 250 mL.
 - f. Funnel: 3 inches diameter.
 - g. Filter paper.
-

DETERMINATIVE METHOD

C. REAGENTS AND SOLUTIONS

**Reagent and
Solution List**

-
- a. Sodium hydroxide 2N: Dissolve 80 g NaOH in 600 mL of water and make to 1 L.
 - b. Citrate buffer solution: Dissolve 117.6 g of citric acid in 560 mL of 2N NaOH and make up to 1 L with water. Keep refrigerated.
 - c. Phosphoric acid-meta, 6%: Fisher A-280 or equivalent. Stable for one week at 4° C.
 - d. Ascorbic acid: Merck 00845-61288 or equivalent.
 - e. 2,6-Dichloroindophenol sodium salt. Fisher S-286 or equivalent: Dissolve 100 mg of dye in boiled distilled water and make up to 100 mL. Take 4 mL aliquot of this solution and make up to 250 mL with water. Keep refrigerated. Prepare weekly.
 - f. Phosphoric acid-meta, 3%.
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DETERMINATIVE METHOD

D. STANDARDS

Preparation of Standards

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- a. Vitamin C standard A: Weigh 100 mg of ascorbic acid into a 100 mL plastic beaker. Add 50 mL of 6% m-HPO₃, and dissolve. Transfer to a 100 mL volumetric flask and make up to volume with water.
 - b. Vitamin C standard B: Transfer a 5 mL aliquot of solution A into a 100 mL volumetric flask, and make up to volume with 3% m-HPO₃.
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DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE

Determination

- a. Weigh 300 g of sample into beaker and transfer to blender.
- b. Weigh 300 g of 6% m-HPO₃ into beaker and transfer to blender. Mix the sample with the m-HPO₃ present. This keeps the ascorbic acid from oxidizing.
- c. From this mixture, weigh 20 g and transfer to a 100 mL volumetric flask.
- d. Add 40 mL of 6% m-HPO₃, and make up to volume with H₂O. This gives a 3% solution of m-HPO₃.
- e. Filter the solution into a 250 mL Erlenmeyer flask.
- f. From the clear filtrate, take an appropriate aliquot (10 mL) and transfer to a 25 mL volumetric flask.
- g. Add 5 mL of buffer solution and make up to volume with 3% m-HPO₃.
- h. The final concentration of the solution should be 3 µg/mL and be in a volume of 25 to 50 mL, but no more.
- i. Prepare standard solution C to be read on the spectrophotometer by transferring a 3 mL aliquot of solution B to a 50 mL volumetric flask.
- j. Add 10 mL of buffer solution and make up to volume with 3% m-HPO₃.
- k. Prepare the blank by transferring 5 mL of buffer solution to a 25 mL volumetric flask and make up to volume with 3% m-HPO₃.

The samples, the standard, and the blank should be run in duplicate, so enough test tubes to accommodate these should be placed in the test tube rack.

The readings on the spectrophotometer should be taken at 515 nm. Place distilled water in one of the test tubes and set the instrument to 100% transmittance.

- l. Into each of the test tubes, pipet 3 mL of dye.
- m. Pipet 3 mL of standard solution C to the dye in one of the test tubes and read. The addition should be made rapidly. Color changes from blue to pink.
- n. Add a few crystals of ascorbic acid to the test tube and read. The solution is reduced and becomes colorless. Do this to a duplicate tube and record readings.
- o. Pipet 3 mL of sample to tube and read. Reduce with a few crystals of ascorbic acid and read. Do this to duplicate tube and record readings.
- p. Pipet 3 mL of blank solution to each of two test tubes.
- q. To one of the test tubes, add a few crystals of ascorbic acid; to the other tube, add nothing.

DETERMINATIVE METHOD

F. ANALYTICAL PROCEDURE (Continued)

- r. Set the blank with the ascorbic acid to the reading obtained when the standard was reduced (e.g., 0.013) and then read the other blank (e.g., 0.357).
- s. This is the corrected reading and is substituted for the reduced reading. Do this for all the reduced readings and record the corrected reading.

Example

<i>Standard Solution C</i>	<i>Duplicate</i>		<i>Average</i>	
0.201	0.201	=	0.201	unreduced standard reading
0.013	0.009	=	0.011	reduced standard reading
0.188	0.192	=	0.190	corrected standard reading
<i>Sample Solution</i>	<i>Duplicate</i>		<i>Average</i>	
0.284	0.292	=	0.288	unreduced sample reading
0.018	0.018	=	0.018	reduced sample reading
0.266	0.274	=	0.270	corrected sample reading

DETERMINATIVE METHOD

G. CALCULATIONS

1. Procedure

United States Recommended Daily Allowance (USRDA) = 60 mg

$$R \times K \times \frac{Df}{1000} \times F = \text{mg/serving}$$

$$\% \text{ USRDA} = \frac{\text{mg/serving}}{60} \times 100$$

R = (corrected dye blank – corrected sample reading)
(corrected dye blank obtained in section F, step r.)

$$K = \frac{\text{Concentration of standard}^*}{(\text{corrected dye blank} - \text{corrected standard reading})}$$

* Concentration of standard is 9.0 μg if method is followed.

$$Df = \frac{10 \text{ g}}{100 \text{ mL}} \times \frac{10 \text{ mL}}{25 \text{ mL}} \times 3 \text{ mL} = 0.12 \text{ g} = 120 \text{ mg}$$

F = Serving size converted to the appropriate dimensions
(i.e., ounces to grams, etc.)

2. Reference

Methods of Vitamin Assay, The Association of Vitamin Chemists (1966).
